Chlamydia pneumoniae and vascular diseases

Maraha, B.

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Boulos Maraha

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Boulos Maraha

geboren te Kamechli, Syrië

promotor: prof.dr. C.M.J.E. Vandenbroucke-Grauls
copromotor: dr. M.F. Peeters

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Chapter 1

Introduction

Introduction

For decades research on the pathogenesis of vascular disease has been focused on classical risk factors, including hyperlipidemia, hypertension, smoking, diabetes, sex, age, and familial history. However, not all cases can be explained by these well-defined risk factors. Therefore, the search for novel potential risk factors is continuing. A central role for inflammation in atherogenesis has been established. Current evidence indicates that inflammatory processes are implicated in the initiation and the evolution of the atherosclerotic process [22, 31]. The initial step in atherosclerosis is probably endothelial dysfunction, which may be caused by a risk factor or a combination of several risk factors [31]. Various infectious pathogens, including *Helicobacter pylori*, cytomegalovirus, herpes simplex virus, and *Chlamydia (C.) pneumoniae,* have been considered as potential risk factors for vascular diseases.

The hypothesis that infection might play a role in the development of vascular diseases supposes that infectious agents, such as *C. pneumoniae*, initiate and progress inflammation, which may contribute to the development of vascular disease [11, 18, 31]. After respiratory tract infection, *C. pneumoniae* can reach vascular tissue via infected leukocytes, where it can infect cells associated with atherosclerosis (endothelial cells, macrophages and smooth muscle cells). Chlamydial lipopolysaccharide and chlamydial heat shock protein 60 kd (cHsp60) may contribute to atherogenesis in several ways [18]. Lipopolysaccharide mediates ingestion of low-density lipoprotein (LDL) by macrophages infected with *C. pneumoniae*, leading to the formation of foam cells, the characteristic cells of early atherosclerosis. CHsp60 mediates oxidation of lipoproteins, which become atherogenic. CHsp60 may also cause proinflammatory activation, which promotes atherogenesis [18]. It has been suggested that cHsp60 may induce immunological cross-reaction with autoantigens such as human Hsp60 leading to antibody-mediated endothelial cytotoxicity [26]. Moreover, infected atheroma-associated cells, such as endothelial cells, seem to produce inflammatory cytokines and express leukocyte adhesion molecules. Endothelial infection may stimulate smooth muscle cells proliferation, whereas infection of macrophages and smooth muscle cells induces production of inflammatory cytokines [18]. It has been suggested that *C. pneumoniae* may cause impaired arterial relaxation and endothelial dysfunction [23]. Moreover, a role for *C. pneumoniae* infection in plaque destabilization has been postulated; it may promote the secretion of matrix-degrading metalloproteinases that destabilize the atherosclerotic plaque [18].

Since the reported association between *C. pneumoniae* and coronary artery disease in 1988, the theory of *C. pneumoniae* as a cause of vascular disease has received considerable attention [33]. Many investigations have addressed the possible involvement of *C. pneumoniae* in vascular diseases [19, 21]. Mice and rabbit models of *C. pneumoniae* infection have been used in the evaluation of the association between *C. pneumoniae* and vascular diseases. Systemic dissemination of *C. pneumoniae* after respiratory infection has been shown in animal model studies. Some animal experiments suggested a role for *C. pneumoniae* in initiation and

progression of atherosclerosis-like inflammatory changes, however they did not establish an etiologic role of *C. pneumoniae* in vascular disease [7, 27-29]. Two antibiotic trials in rabbits showed that *C. pneumoniae* increased intimal thickness and induced atherosclerotic changes [8, 29]. Early administration of antibiotic therapy reduced these atherogenesis effects of *C. pneumoniae*, however delayed treatment (weeks after infection) was ineffective. It has been shown that antibiotic treatment did not affect the presence of *C. pneumoniae* in rabbits and mice [29, 32]. The results of two recent studies in mice and rabbits questioned the effect of antibiotic treatment. In a mice model, antibiotic treatment had no effect on atherosclerotic changes caused by *C. pneumoniae* at all [32]. Antibiotic treatment had, in a rabbit model, no reducing effect on the prevalence of atherosclerotic lesions but it reduces only the extent of the lesions [9]. Furthermore, it has to be mentioned that rabbit and mice models of atherosclerosis are not identical to that of human atherosclerosis. Another limitation of experimental animal models is that investigations have been limited to rabbit and mice [10].

The promising preliminary results of two small human clinical trials, published in 1997, increased the enthusiasm for the hypothesis that links *C. pneumoniae* to vascular diseases [12, 13]. Gupta et al. [12] randomized 60 patients with myocardial infarction and positive *C. pneumoniae* serology (IgG $\geq$ 64) to receive either a daily dose of oral azithromycin 500 mg or a placebo for 3 or 6 days. Patients in the azithromycin group had lower risk of subsequent cardiovascular events after a follow-up period of 18 months. Also, a decrease in IgG titers against *C. pneumoniae* was found in the azithromycin group. Gurfinkel et al. [13] randomized 202 patients with unstable angina or non-Q-wave myocardial infarction to receive oral roxithromycin 150 mg twice daily or placebo for 30 days. A significant reduction in subsequent cardiovascular events was found in the roxithromycin group after 1-month follow-up. However, this reduction was no more significant after 6 months follow-up [14]. Furthermore, IgG titers against *C. pneumoniae* were not influenced by roxithromycin.

Atherosclerosis

Atherosclerosis evolves in the arterial intima as a result of proliferation of smooth muscle cells and accumulation of macrophages, lymphocytes and lipids [31]. As the process advances, the atherosclerotic plaque is formed containing smooth muscle cells, collagen, elastic fibers, macrophages, lymphocytes, lipids, cellular debris, and calcification. In a more advanced stage, the endothelium surface of the lesion is damaged; the plaque becomes vascularized leading to hemorrhage and thrombosis formation. Finally, the arterial vessel is occluded.

The pathogenesis of atherosclerosis is not fully understood. Several hypotheses have been proposed to explain the etiology of this disease. The widely accepted ‘response to injury’ hypothesis postulates that atherogenesis probably starts with endothelial dysfunction as a result of exposure to elevated modified LDL, free radicals originated by tobacco abuse, diabetes mellitus, elevated homocysteine, genetic mutation and possibly other risk factors not

defined yet [31]. Endothelial dysfunction leads to the production of adhesion-molecules that increase adherence and invasion of monocytes and T lymphocytes into the arterial intima. Subsequently, monocytes/macrophages accumulate LDL and form foam cells. Locally produced cytokines and growth factors progress the lesion by stimulating the migration of smooth muscle cells from arterial tunica to arterial intima, where they proliferate. The involvement of inflammatory responses, including inflammatory cells (macrophages and T lymphocytes) and cytokines in all stages of the atherosclerotic process indicates a central role for inflammation in atherosclerosis [31].

Abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) is a localized chronic dilatation in the abdominal aorta. It results from genetic and acquired weakness in the arterial media. Degradation of extracellular matrix proteins is the most important feature in the pathogenesis of AAA, leading to fragmentation of elastin and collagen fibers in the aortic wall and subsequently resulting in expansion of the arterial wall [24]. There is evidence that genetic factors are implicated in the development of AAA. This is supported by the familial clustering of AAA. Also, several proteolytic factors are considered as risk factors for AAA. Interaction between these risk factors probably promotes proteolytic activity in the arterial wall, which gives rise to aneurysmal dilatation [17, 24]. However, the pathogenesis of AAA is not fully understood and efforts to identify novel risk factors are continuing.

Venous thrombosis

Venous thrombosis affects mainly the deep venous system of the legs. Factors that are involved in the pathogenesis of venous thrombosis include endothelial injury, stasis and hypercoagulation. Adherence of the thrombus to the vascular endothelium characterizes venous thrombosis. The thrombus contains coagulated blood, platelets, fibrin and cellular components. Acquired and genetic risk factors contribute to the development of venous thrombosis [30]. Acquired risk factors include immobilization, trauma, surgery, pregnancy, malignancy and female hormones. Genetic factors include coagulation abnormalities such as factor V Leiden, protein C deficiency, protein S deficiency, antithrombin deficiency, prothrombin 20210A, hyperhomocysteinaemia and high level of factor VIII. However, about one-third of episodes of venous thrombosis cannot be explained by the established risk factors.

***Chlamydia pneumoniae* (*Chlamydophila pneumoniae*)**

According to the "old" classification of the order *Chlamydiales*, the family *Chlamydiaceae* contained only the genus *Chlamydia,* and this genus had four species: *C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum*. Recently a new classification of the order *Chlamydiales* has been presented (figure 1). In the new classification five new species (*suis, muridarum, abortus, felis* and *caviae*) are added and *C. pneumoniae*, *C. pecorum* and *C. psittaci* are moved to the new genus *Chlamydophila* [6]. The separation of *Chlamydia* and *Chlamydophila* is based on differences in genome size and protein sequence analysis. In addition, in contrast to *Chlamydia* species, *Chlamydophila* species do not produce detectable glycogen and have one ribosomal operon (*Chlamydia* species have two). Furthermore, three new non *Chlamydiaceae* families have been added, the *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae*. A number of eminent chlamidiologists are against the new classification [34]. According to these chlamidiologists there is insufficient reason to divide the *Chlamydiaceae* into 2 genera. In this thesis, we did not use the new genus name *Chlamydophila*, since microbiologists and clinicians are used to the "old" genus name *Chlamydia*.

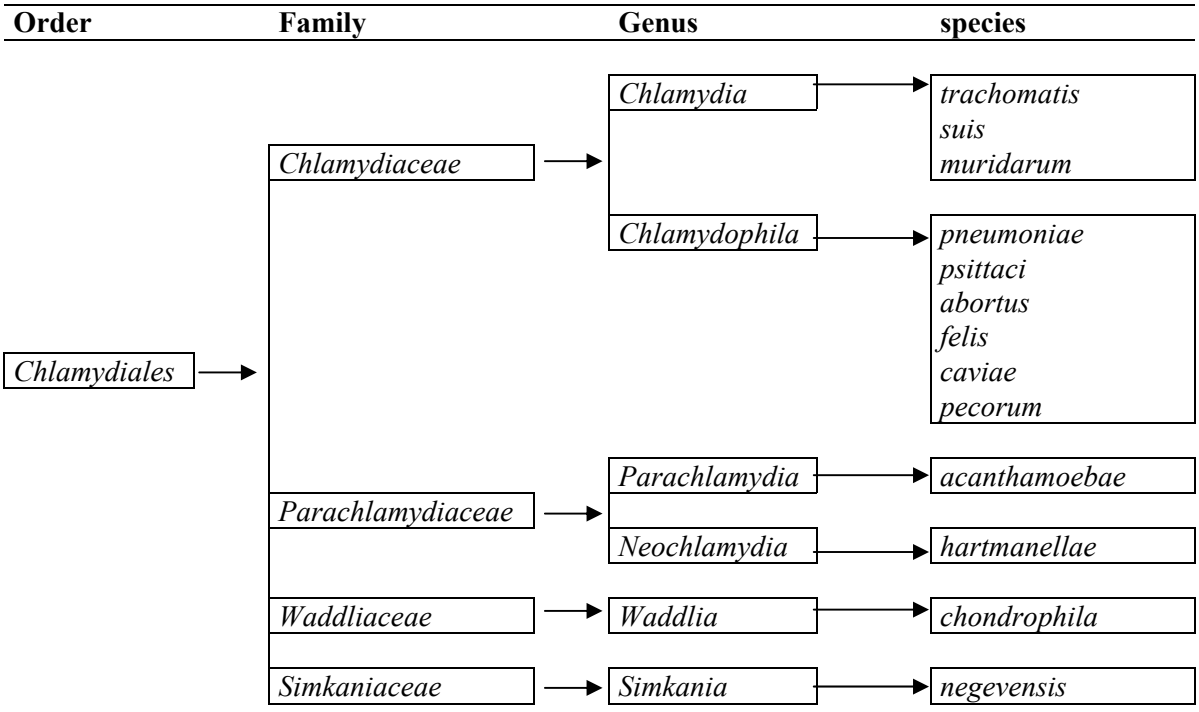

Figure 1. The new classification of the order *Chlamydiales.*

C. pneumoniae is a Gram-negative obligate intracellular bacterium with a biphasic life cycle (figure 2). A smaller extracellular infectious form called the elementary body and a larger replicating intracellular non-infectious form called the reticulate body characterize the development cycle of *Chlamydiaceae*. After attachment to host cells, elementary bodies enter the cell, probably by endocytosis, and differentiate into reticulate bodies. Reticulate bodies

replicate, using the host cell energy, and form inclusions. Prior to cell lysis and release from the host cell, reticulate bodies transform again into elementary bodies [20]. This biphasic life cycle lasts for 48 to 72 hours.

C. pneumoniae DNA shows little homology with *C. trachomatis, C. psittaci* and *C. pecorum*, less than 5%, 10% and 10% respectively [20]. Moreover, elementary bodies of *C. pneumoniae* are pear-shaped, whereas those of *C. trachomatis, C. psittaci* and *C. pecorum* are round-shaped. *C. pneumoniae* and *C. trachomatis* have no animal reservoir and humans are the only known reservoir, whereas *C. psittaci* and *C. pecorum* have an animal reservoir. The natural host of *C. psittaci* is birds and lower mammals, whereas cattle and sheep are the natural reservoir of *C. pecorum*.

Transmission of *C. pneumoniae* occurs from person to person probably via respiratory secretions. Although survival of *C. pneumoniae* on surfaces is very short, transmission via this way might be also possible. *C. pneumoniae* infection has an incubation period of 7-21 days. This infection is endemic, however, epidemics that last for several months occur every 2-4 years. Outbreaks have been reported in schools, military bases and nursing homes [2, 5, 35].

In contrast to other chlamydial species, the major outer membrane protein (MOMP) of *C. pneumoniae* is not immunodominant and does not contain species-specific antigens [20]. Therefore, reactivity to the MOMP is cross-reactive among chlamydial species. The immunodominant *C. pneumoniae*-specific 98-kDa protein seems to be present only in the outer membrane complex of *C. pneumoniae*. Other *C. pneumoniae*-specific proteins include the 43-kDa protein and proteins with molecular mass between 50 and 60 kDa [20].

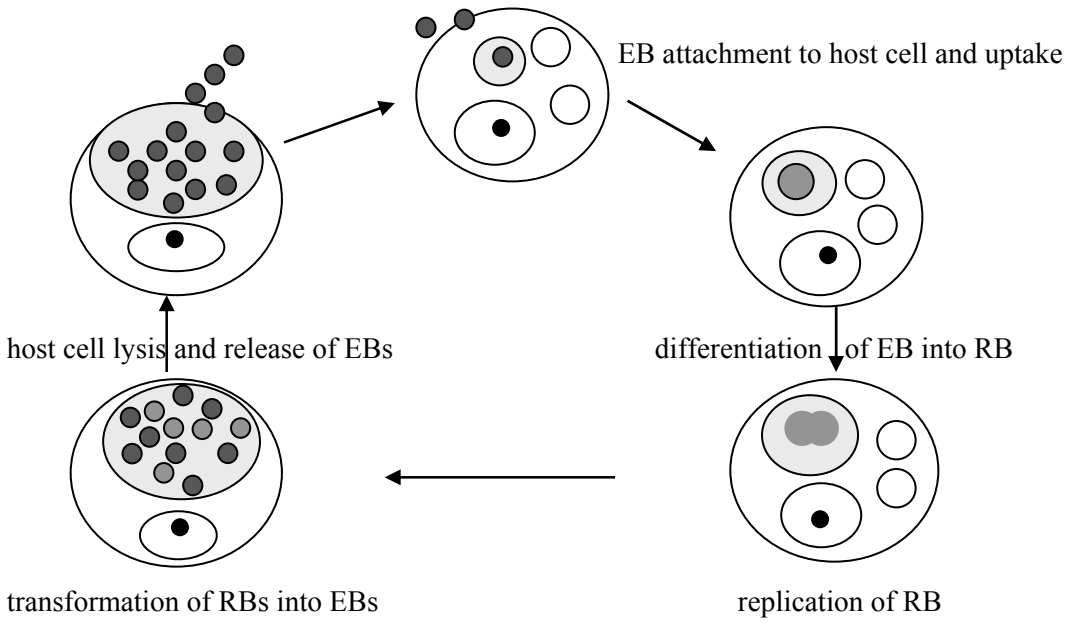

Figure 2. The development cycle of *C. pneumoniae*. EB, elementary body; RB, reticulate body.

Infection with *C. pneumoniae* induces serum immunoglobulin responses including IgM, IgA, and IgG. In primary infection, IgM response appears within 3 weeks and IgG response after 6 to 8 weeks. In re-infection, IgM response may be absent and the IgG response occurs within 1 to 2 weeks [16]. The biological half-life of serum IgA is about 7 days, whereas the half-life of IgG is 23 days [16]. Therefore, it has been suggested that the persistence of positive IgA titer can be used as a marker for chronic infection. However, the Centers of Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) recommend not to use neither elevated IgA nor any other serologic markers as criteria for chronic or persistent infection [4]. It has been suggested that high levels of IgG interfere with determination of IgA titers [3]. In a serological study IgA was detectable in only 17 to 42% of patients with serological evidence (IgG and /or IgM) for acute or recent infection [36].

The seroprevalence of *C. pneumoniae*, determined by the MIF test, increases from 10% at the age of 5-10 years to 50% of adults (> 20 years) reaching 70-80% among persons above the age of 50 years [15]. Re-infection throughout life with *C. pneumoniae* is probably very common. Seroprevalence is higher in adult males than in adult females; so far, no explanation for this has been found. However, there is no difference in seroprevalence between sexes under 15 years of age.

C. pneumoniae, *Mycoplasma pneumoniae* and *Legionella pneumophila* cause community-acquired pneumonia. However, the data on the significance of these bacteria as agents of community-acquired pneumonia are inconsistent. According to different studies, these pathogens are responsible for 2-30% of community-acquired pneumonia [15]. It has been demonstrated that these rates are influenced by the diagnostic serologic criteria used [25]. *C. pneumoniae* is also associated with acute bronchitis and pharingitis. As with community-acquired pneumonia, conflicting data have been reported on the rate of *C. pneumoniae* as the agent of acute bronchitis (range 2% - 25%) [1]. These data depend on the diagnostic methods and criteria used.

Outline of the thesis

The aim of this thesis is to assess the possible association between *C. pneumoniae* and vascular diseases.

In chapter 2, four different methods for DNA extraction from vascular tissue are compared. A homogenous solution was prepared from aorta tissue samples inoculated with known concentrations of *C. pneumoniae* DNA. The spiked dilution series were tested by four procedures to extract *C. pneumoniae* DNA. Extracted DNA was detected by polymerase chain reaction (PCR).

In a case-control study (chapter 3), the association between abdominal aortic aneurysm (AAA) and *C. pneumoniae* is investigated. Using PCR, we explored the presence of *C. pneumoniae* DNA in peripheral blood cells samples of patients with AAA and control

subjects. Also, the seroepidemiologic association between AAA and *C. pneumoniae* was investigated.

In chapter 4, the impact of serologic methodology on the association between *C. pneumoniae* and AAA is assessed. The association between *C. pneumoniae* and AAA was investigated by five serologic tests. In addition, the agreement between these tests was evaluated.

We studied, in chapter 5, the involvement of *C. pneumoniae* in the inflammation associated with venous thrombosis. Using PCR and serology, the association between *C. pneumoniae* and venous thrombosis was assessed in a case-control study.

In chapter 6, the association between *C. pneumoniae* and atherosclerosis is assessed. Also, the correlation between the detection of *C. pneumoniae* by PCR and immunohistochemical staining (IHC) in vascular specimens was evaluated. The correlation between *C. pneumoniae* serology and the detection of this pathogen was analyzed.

Chapter 7 deals with the hypothesis that not only *C. pneumoniae* but also *Mycoplasma pneumoniae* is a plausible candidate to play a role in the pathogenesis of atherosclerosis, because of its ability to induce chronic inflammation and its epidemiological behavior that simulates the epidemiological behavior of *C. pneumoniae.* We investigated, by PCR, the presence of *M. pneumoniae* in atheromas and in degenerative heart valve specimens obtained from patients undergoing vascular surgery.

Chapter 8 addresses the impact of molecular methodological factors on the association between *C. pneumoniae* and vascular disease. Vascular specimens were tested by three PCR assays: a 16S PCR-based reverse line blot assay, a single-step PCR and a nested PCR. We, also, explored the impact of hybridization and the use of different DNA polymerase enzymes on the results of the PCR assays.

In chapter 9, a randomized, double-blind, placebo-controlled trial is described. In this trial, we investigated the effect of clarithromycin on the presence of *C. pneumoniae* in vascular tissue of patients with coronary artery disease. Also, the effect of clarithromycin on *Chlamydia* IgG titers was evaluated. IHC, a real-time PCR and an industry-developed research-use-only PCR assay were used to assess the eradication of *C. pneumoniae* from vascular specimens obtained from the study population during coronary artery bypass graft surgery.

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Chapter 2

Extraction of *Chlamydia pneumoniae* DNA from vascular tissue for use in PCR: an evaluation of four procedures

Hans Berg, Boulos Maraha, Anneke Bergmans, Anneke van der Zee, Jan Kluytmans, Marcel Peeters

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Abstract

The objective of this study was to compare four procedures for *Chlamydia pneumoniae* DNA extraction from vascular tissue. The NucliSens Kit, the QIAamp tissue DNA MiniKit, buffer-saturated phenol and the Geneclean II Kit were evaluated, based on the yield of recovered DNA, using PCR to detect *C. pneumoniae* in vascular tissue. The QIAamp tissue procedure had the highest detection level (0.004 inclusion-forming units/sample). All methods, except NucliSens (70 min), had a short handling time (30–40 min). Costs varied from 0.5 to 3.2 euro.

Introduction

Chlamydia pneumoniae has been associated with atherosclerosis, initially on the basis of seroepidemiologic studies [1, 2]. Two recent studies, however, have failed to demonstrate any such connection [3, 4]. Subsequently, further evidence has been provided by the detection of *C. pneumoniae* in atherosclerotic tissue by polymerase chain reaction (PCR), immunocytochemistry [5–7], and isolation in culture. Whether *C. pneumoniae* plays a role in the pathogenesis of atherosclerosis is still not known. Although serologic assays are considered the reference diagnostic method for *C. pneumoniae* infections, PCR is potentially an important tool for further studies in this field. *C. pneumoniae* PCR is, however, not yet standardized. Several PCR assays have been used to detect *C. pneumoniae* in vascular tissues. However, a considerable variation in the detection rate of *C. pneumoniae*, ranging from 0% to 100%, has been reported by different investigators [5–10]. This phenomenon can be explained by the differences between the populations studied, by sampling error, and by differences in the PCR techniques applied. The potential for differences in performance between PCR techniques is well known. For example, the sensitivity of PCR for the detection of *Mycobacterium tuberculosis* in samples containing low numbers of microorganisms varied among seven laboratories from 2% to 90% [11]. A multicenter study showed major inter-laboratory differences in the detection rate of *C. pneumoniae* in endarterectomy specimens [12]. Therefore, it is important to determine a more standardized procedure that includes DNA extraction from specimens. In order to detect *C. pneumoniae* by PCR, efficient release of *C. pneumoniae* DNA from vascular tissue and adequate removal of PCR inhibitors (lipids and calcification) are essential [13]. The purpose of this study is to compare four procedures for *C. pneumoniae* DNA extraction from vascular tissue.

Materials and Methods

Vascular samples were obtained from 30 patients during cardiac surgery (coronary artery bypass graft—CABG). During this procedure, a punch biopsy through the aortic wall is routinely taken. This biopsy material was stored at 4 ^{o}C in 200 µL of lysis buffer (1M Tris, pH 7.0, 0.5 mM EDTA, 5 M NaCl, 1% sodium docecyl sulfate (SDS), 20mg/mL proteinase K) for a maximum of 24 h. Subsequently, the sample was lyzed by adding 20 µL of proteinase K (20 mg/mL, Qiagen, Hilden, Germany) and incubating overnight at 56 ^{o}C. After cell lysis, a

homogeneous solution was made by pooling all 30 lysates. Seven 900-µL portions of the homogenate were inoculated with decreasing concentrations of inclusion-forming units (IFUs) of *C. pneumoniae* strain TW 183. Portion number 8, containing AE buffer (QIAamp, Qiagen), was used as a negative control.

Two hundred and five microliters of each portion (corresponding to the concentrations of the dilution series (10- and 5-fold): 10 000, 1000, 100, 10, and 2, 0.4, 0.08 IFU per 205 µL; per PCR (this is 500, 50, 5, 0.5, 0.1, 0.02 and 0.004 IFU) was subjected to each of four methods of DNA extraction.

In the first procedure NucliSens (Organon Teknika, Boxtel, The Netherlands) used, based on the method of Boom et al. [14], according to the manufacturer's instructions. This technique is based on the mechanism whereby DNA binds to glass particles (silica) in a high concentration of chaotropic salt, while contaminants such as proteins, carbohydrates and ions do not. A wash procedure is repeated three times to remove all contaminants (with wash buffer, or 70% ethanol or acetone). DNA is eluted from the silica by resuspension of the silica complexes in NucliSens elution buffer.

In the second procedure the QIAamp DNA MiniKit (Qiagen) was used, according to the QIAamp tissue protocol in the manufacturer's instructions. This method uses a QIAamp spin column to which DNA binds in the presence of buffer AL and ethanol. Two wash steps, in which AW1 buffer and AW2 buffer succeed each other, are performed to remove contaminants. AE buffer is finally used for elution of the DNA from the spin column.

In the third procedure buffer-saturated phenol (Life Technologies, Breda, the Netherlands) was used. This method is home-made, based on 'classical' phenol extraction. In this method, 200 µL of buffer-saturated phenol (pH 7.5–7.8) was added to 205 µL of sample in a 1.5-mL screw-cap plastic tube, vortexed for 1 min, and centrifuged for 5 min at 20 000 g. The aqueous supernatant was transferred to another tube, which also contained 200 µL of buffer-saturated phenol. After homogenizing and centrifuging (5 min at 20 000 g), the aqueous supernatant was transferred to a 1.5-mL screw-cap plastic tube containing 20 µL of 3M sodium acetate (pH 5.2) and vortexed. Ice-cold absolute ethanol (440 µL) was added, and the mixture was homogenized and incubated at -20 ^{o}C for 15 min. The sample was centrifuged (15 min, 20 000 g) to pellet the DNA products. The supernatant was removed, and the pellet resuspended in 250 µL of 70% ethanol, vortexed and centrifuged for 5 min (20000 g). The supernatant was removed again, and a quick centrifuge spin was done, so that the remaining ethanol could be removed. The pellet was air-dried in a half-open tube and suspended in 100 µL of AE buffer by vortexing.

In the fourth procedure the Geneclean II Kit (Qbiogene, Illkirch, France) was used, according to the manufacturer's instructions. Like the first method, this method is also based on the fact that DNA binds to glass particles in a high concentration of chaotropic salt. Here, the DNA binds to Glassmilk. The Glassmilk–DNA pellet is washed once with New Wash.

All DNA elutions (in 100 µL of AE buffer, (Qiagen)) were resuspended at 80 ^{o}C for 5 min, which completed the DNA extraction.

An identical PCR assay was performed on 5 µL of each sample to detect *C. pneumoniae* DNA, irrespective of extraction method. All amplification steps, assay conditions, signals, visualization steps and hybridization procedures were identical.

Polymerase chain reaction

Primers CpnA (5'-TGA CAA CTG TAG AAA TAC AGC-3') and CpnB (5'-CGC CTC TCT CCT ATA AAT-3') were used in a PCR based on the 16S rRNA gene sequence as described by Gaydos et al. [15].

The PCR reaction mixture contained 30 pmol of each primer, 3mM $MgCl_2$, 200 µM dNTPs (dTTP is replaced by dUTP), 2.5 units of Ampli*Taq* Gold DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA), 10 x PCR buffer II (Perkin Elmer), and 1.25 µL of internal control.

An internal control was added in each reaction to enable detection of inhibition of the PCR reaction and prevent false-negative PCR results. The internal control template DNA consisted of a PCR product of an unknown fragment of *Escherichia coli* DNA that yields a 150-bp PCR product in combination with primer PINTK (5'-(ACTG x 4)-AC-3').

The PCR amplification was performed as follows: after addition of 5 µL of template DNA in a final volume of 25 µL of PCR reaction mixture, samples were subjected to the following PCR program: 10 min at 96 ^{o}C, followed by 40 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C, and 1 min at 72 ^{o}C. A final step of 10 min at 72 ^{o}C completed the PCR in a thermocycler (9600, Perkin Elmer). A negative PCR mix control and a negative sample-processing control were included in each PCR run with every five samples to detect false-positive results.

For final product detection, amplification products were examined by agarose gel electrophoresis and dot-blot hybridization as follows. Eight microliters of each PCR product was analyzed by agarose gel electrophoresis on 2% agarose gels in TBE buffer containing ethidium bromide. PCR products were visualized under UV transillumination and photographed. If the 450-bp *Chlamydia*-derived band was visible (with or without the 150-bp band), the sample was considered positive. If only the 150-bp band of the internal control was visible, the sample was considered negative. If no bands were visible, the PCR was considered inhibited, and the sample was repurified and retested by PCR.

Dot-blot hybridization

Hybridization of 5 µL of the PCR products was performed using a 5'-biotinylated *C. pneumoniae*-specific probe, Cpneu-B: 5'-ACACACGTGCTACAATGGTT-3'. Hybridization signals were visualized using streptavidin peroxidase (Boehringer Mannheim, Mannheim, Germany) and ECL detection reagents (Amersham, Biosciences UK limited, Little Chatfont, UK).

To minimize the risk of contamination, sample preparation, PCR amplification and analysis of the PCR product were performed in separate rooms.

Analysis

Comparison of the four procedures was done using gel electrophoresis and dot-blot hybridization. Each procedure was also compared for overall time consumption and hands-on time per sample. Hands-on time was defined as time needed by the technician working with this procedure, and overall time as the total time, including centrifuging, incubation, etc. Finally, the average costs per sample were estimated in euros, calculated from the price of the commercial kit and material (e.g. ethanol), excluding the use of materials such as plastic tubes, divided by the number of samples.

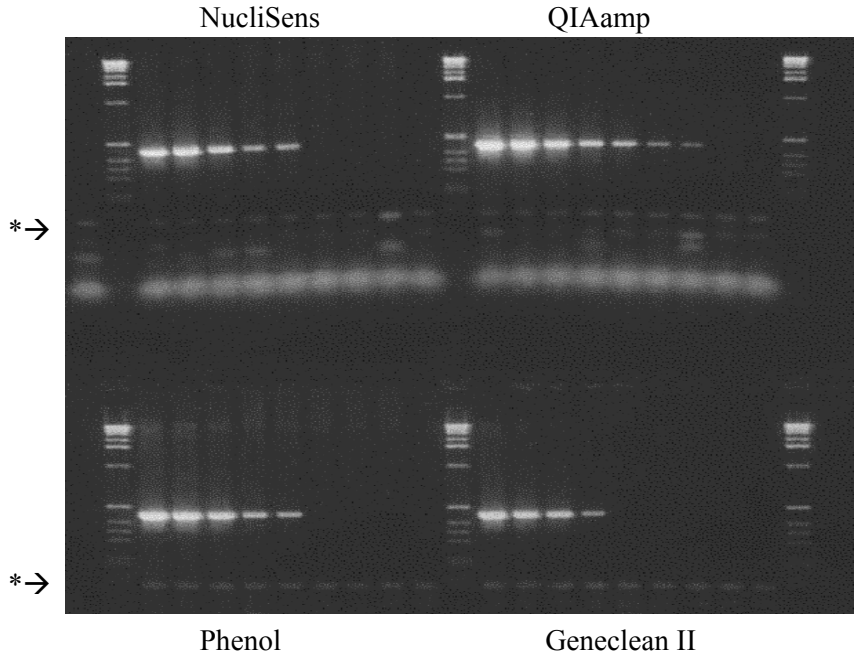

Figure1. Agarose gel electrophoresis of PCR products (*Chlamydia pneumoniae*) using four DNA extraction methods.
* Internal control bands.

Results

Figure 1 shows the results of *C. pneumoniae* detection for each method. The detection levels ranged from 0.004 IFU per sample for QIAamp, to 0.1 IFU per sample for phenol extraction and NucliSens, and 0.5 IFU per sample for Geneclean II (table 1).

The least labor-intensive method was the Geneclean II, with a hands-on time of 30 min, and an overall time of 60 min (table 1). Costs per sample of various methods show that buffer-saturated phenol was the cheapest method, with an average of 0.5 euro per sample (table 1).

Table 1. DNA extraction methods for *Chlamydia pneumoniae* in vascular tissue

Method	Detection level IFU/sample	Time consumption (min)		Costs (euro) per sample
		Hands-on time	Total time	
NucliSens	0.1	70	115	3.2
QIAamp	0.004	35	60	2.9
Phenol	0.1	40	105	0.5
Geneclean II	0.5	30	60	1.4

Discussion

In this study, we compared four different DNA extraction methods. To create realistic study material, a homogenate solution was prepared from aorta tissue samples inoculated with IFUs from *C. pneumoniae*. The QIAamp DNA MiniKit extraction method detected the lowest amount of IFUs by far. Also, it is a rapid and easy-to-perform procedure. The associated costs represent a disadvantage.

Several factors influence the ability of PCR to detect *C. pneumoniae,* including sample preparation, DNA extraction, amplification assays, and visualization procedures. Standardization of these factors was therefore approached in the present study.

Because *C. pneumoniae* is an intracellular pathogen, vascular tissue was treated with proteinase K to produce tissue cell lysis and release *C. pneumoniae* DNA, if present. The aorta samples used in the present study were macroscopically non-atherosclerotic, and the expected positivity rate was low [16]. Since the amount of *C. pneumoniae* in the study materials was unknown, a homogeneous pool of all 30 lysates was made and inoculated with decreasing concentrations of IFUs to enable us to compare DNA extraction methods. It should be mentioned that the concentration of *C. pneumoniae* in the dilution series was very similar, though it is impossible to achieve identical concentrations. Moreover, it is not known whether the ability of the four procedures to extract *C. pneumoniae* DNA from spiked materials is the same as their ability to extract DNA from patient materials [12].

A multicenter study [12] demonstrated that the sensitivity of a PCR assay does not necessarily correspond with the ability to detect *C. pneumoniae* in patient material, without a logical explanation. In the present study, we performed one PCR assay, and because there is

no reference assay available, one should view the results of the present study in the light of the absence of a reference PCR assay.

Taking the limitations of the present study into consideration, we can conclude that QIAamp is a useful and sensitive DNA extraction method, but further effort to optimize and standardize DNA extraction methods is needed.

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Chapter 3

Detection of *Chlamydia pneumoniae* DNA in buffy-coat samples of patients with abdominal aortic aneurysm

Boulos Maraha, Martin den Heijer, Martina Wullink, Anneke van der Zee, Anneke Bergmans, Harold Verbakel, Marjolein Kerver, Sytse Graafsma, Steef Kranendonk, Marcel Peeters

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Abstract

Recent studies have suggested that *Chlamydia pneumoniae* infection is a risk factor for abdominal aortic aneurysm. This study explores the presence of *C. pneumoniae* DNA in buffy-coat samples of control subjects and of patients with abdominal aortic aneurysm. The seroepidemiological association between abdominal aortic aneurysm and *C. pneumoniae* was also investigated. Buffy-coat samples and serum specimens were obtained from 88 patients and 88 control subjects. Detection of *C. pneumoniae* DNA in buffy-coat samples and measurement of IgG antibodies to *C. pneumoniae* in serum specimens were performed by polymerase chain reaction and microimmunofluorescence, respectively. *C. pneumoniae* DNA was detected in buffy-coat samples of 18 (20%) patients and 8 (9%) control subjects (adjusted odds ratio 2.9, 95% confidence interval 1-8.5). IgG antibodies to *C. pneumoniae* were detected in 85 (97%) patients and 71 (81%) control subjects (adjusted odds ratio 7.2, 95% confidence interval 1.7-31). The results show an association abdominal aortic aneurysm and either the presence of *C. pneumoniae* DNA in buffy-coat samples or IgG antibodies to *C. pneumoniae*. These findings support the hypothesis that previous infection with *C. pneumoniae* might be a risk factor for abdominal aortic aneurysm.

Introduction

Several risk factors have been identified as playing a role in the pathogenesis of atherosclerosis, the most important process in cardiovascular diseases. In addition to classic risk factors such as hypercholesterolemia, hypertension and cigarette smoking, other potential risk factors including infectious diseases, have recently gained attention [1]. Atherosclerosis has been considered as the most important risk factor for the development of abdominal aortic aneurysm (AAA). Later studies demonstrated the importance of other factors, including genetic factors, familial clustering and several proteolytic factors that interfere with matrix components of the aortic wall [2].

C. pneumoniae is a common cause of respiratory tract infections and has been linked with atherosclerosis and AAA [1, 3]. Association between seropositivity for *C. pneumoniae* and coronary artery disease or myocardial infarction is reported [1, 4]. However, two recent studies failed to demonstrate such a seroepidemiological relationship [5, 6]. *C. pneumoniae* has been detected by polymerase chain reaction (PCR) assays and immunohistochemistry in arterial atherosclerotic lesions and AAA tissue [3, 7, 8]. To evaluate the clinical effect of antibiotic therapy in patients with coronary artery disease, clinical antibiotic trials have been reported, and several large clinical trials are underway [9]. The preliminary clinical trials concluded that antibiotics lead to reduction in cardiovascular events and in markers of inflammation [10-12].

To identify persons with infected vascular tissues, diseased tissues obtained during surgery have been investigated. It has been suggested that detection of circulating *C. pneumoniae* DNA is a useful and an appropriate assay that can predict vascular infection [13-15]. One

study has reported the detection of *C. pneumoniae* in peripheral blood mononuclear cells (PBMCs) from patients with AAA [13]. However, no case-control studies have been conducted to explore the association between the detection of *C. pneumoniae* DNA in peripheral blood cells and AAA.

We investigated, using PCR, the association between AAA and the presence of *C. pneumoniae* DNA in buffy-coat samples. Serological association was investigated using a microimmunofluorescence (MIF) test.

Materials and Methods

Study Population

The study population included 88 patients with AAA and 88 control subjects. From August 1996 to September 1997, a case-control study was performed to evaluate the role of hyperhomocysteinemia in AAA (unpublished data). Subsequently, the association between *C. pneumoniae* and AAA was investigated in the study population. During the study period, all patients who presented with AAA (ultrasonographically proven infrarenal aortic diameter > 30 mm) or who underwent surgery for AAA at our hospital were invited to participate in this study. The patients included were asked to bring a friend or a neighbor as a control subject. Patients and control subjects were matched for sex and age (± 5 years). Some patients brought a control subject who was not of the same sex and age. These control subjects were matched to other patients. For 38 patients we failed to recruit a control subject using this method. These patients were matched to friends or family members of hospital staff personnel (n = 31) or to patients who visited the internal medicine outpatient clinic for reasons not related to vascular disease (n = 6). Ultrasonography was performed in all control subjects to exclude AAA; if an abdominal aortic diameter of > 30 mm was found, the control subject was not included.

Hypertension was defined as the use of antihypertensive medication or blood pressure >140/90 mmHg. Hypercholesterolemia was defined as the use of cholesterol-lowering medication or serum cholesterol >8 mmol/l. Diabetes mellitus was defined as the use of medication for diabetes mellitus or fasting serum glucose > 7.8 mmol/l.

Buffy-coat samples and serum specimens were obtained from patients and control subjects. The study was approved by the local ethical committee, and all subjects gave informed consent.

Sample Preparation and Polymerase Chain Reaction

Separation of buffy-coats from blood samples and DNA extraction were performed on the same day the samples were received. Buffy-coat samples were prepared by centrifuging EDTA-whole blood 3300 xg for 10 min. PCR samples were prepared by extracting total DNA from 200 µl of the buffy-coat fraction using the QIAamp DNA minikit (Qiagen, Germany). Processed specimens were stored at 4 °C for 12 months before the PCR assay was done. As

template in PCR, 5 µl of a 200 µl elution sample was used. Buffy-coat samples of 88 patients and 88 control subjects were examined by PCR for the presence of *C. pneumoniae* DNA using a validated PCR assay as described by Gaydos et al. [16]. The primers CpnA (5'-TGA CAA CTG TAG AAA TAC AGC-3') and CpnB (5'-CGC CTC TCT CCT ATA AAT-3') were used in a PCR assay based on the sequence of the 16S rRNA gene. The sensitivity of the PCR assay was experimentally determined by spiking known concentrations of *C. pneumoniae* DNA in a pool of negative clinical material; the lowest detection limit was 0.1 inclusion-forming unit, indicating good sensitivity. A negative control, containing all PCR reagents without specimens, was processed with every five samples. To control for inhibition of the PCR reaction, a second sample of each buffy-coat sample was spiked with *C. pneumoniae* target DNA. If the spiked sample was negative, the sample was considered inhibited. Inhibited PCR samples were repurified and retested using 2.5 µl of the samples. To minimize the risk of contamination, sample processing and the PCR assay were performed in separate rooms, and to prevent carryover of previous PCRs, uracil DNA glycosylase (UDG) and deoxyuridine triphosphate (dUTP) were used.

PCR products were examined visually after electrophoresis in 2% ethidium bromide-stained agarose gels. To confirm positive results, 3 µl of the PCR product was spotted and hybridized with a 21 bp 5'-biotinylated probe CPNEU-B (5'-GAC ACA CGT ACA ATG GTT-3'). Hybridization signals were examined visually using streptavidin-peroxidase and enhanced chemiluminescence detection reagents (Amersham, UK).

Serological investigations

Ten microlitres of the serum fraction was used for serological investigations. Serum specimens were tested for the presence of IgG antibodies against *C. pneumoniae* (TW 183). The serological testing was performed by a MIF test (MRL Diagnostics, USA) as described previously [17, 18]. An IgG antibody titer $\geq$ 1:16 was considered positive. All serological tests were performed blindly by the same technician.

Statistical Analysis

Matched odds ratios were calculated as an estimate of the relative risk for AAA in subjects with positive *C. pneumoniae* PCR and positive serological results. Using SPSS software (SPSS, USA), confidence intervals and adjusted odds ratios were calculated and a multivariate analysis was performed in order to adjust for other cardiovascular, cerebrovascular and peripheral vascular diseases and for classic risk factors of atherosclerosis.

Association between circulating *C. pneumoniae* DNA and IgG antibodies was analyzed by the chi-square test. The *t* test for two independent samples with equal variance was used to analyze the association between pack-years of cigarette smoking (A pack-year was defined as smoking 20 cigarettes/day for 1 year) and circulating *C. pneumoniae* DNA and IgG

antibodies, and to compare the geometric means titers (GMTs) of patients with those of control subjects.

Results

During the study period, 149 consecutive patients with AAA were invited to participate in the study, but only 89 took part. Eight control subjects were excluded because of asymptomatic AAA. For one patient, aged 94 years, we failed to find a control subject. Thus, the study population consisted of 88 cases and 88 controls. Further characteristics of the study population are shown in table 1.

Table 1. Baseline characteristics of patients with abdominal aortic aneurysm and controls

Characteristic	Patients (n = 88)	Control subjects (n = 88)	P value
Age in years[a] (range)	69 (45-85)	67 (44-83)	NS [c]
Male/female[a]	81/7	81/7	NS
Aneurysm			
Under control	35		
Operated	53		
Elective	40		
Symptomatic	7		
Ruptured	6		
Family history of aneurysm	16	9	NS
History			
Myocardial infarction	26	9	<0.01
Cerebrovascular disease	9	4	NS
Pulmonary embolism	2	3	NS
Chronic obstructive pulmonary disease	24	13	NS
Peripheral vascular disease	19	4	<0.01
Diabetes mellitus	6	4	NS
Smoking			
Pack-years of cigarette smoking[b] (±SD)	34 (±33)	23 (±22)	<0.01
Medication			
Antihypertensive drugs	48	28	<0.01
Cholesterol-lowering drugs	28	8	<0.001
Nonsteroidal anti-inflammatory drugs	37	17	<0.01
Mean systolic blood pressure, mmHg (SEM)	159 (2.4)	158 (2.6)	NS
Mean diastolic blood pressure, mmHg (SEM)	85 (1.2)	87 (1.2)	NS
Mean cholesterol, mmol/l (SEM)	5.7 (1.13)	5.7 (0.10)	NS

[a] Patients and controls were matched for age and sex; [b] A pack-year was defined as smoking 20 cigarettes/day for 1 year; [c] NS, not significant.

Buffy-coat samples from patients and controls were tested for the presence of *C. pneumoniae* DNA. Samples from three patients and two controls showed PCR inhibition. When these five samples were retested using 2.5 µl of the samples, no PCR inhibition was found. PCR detected *C. pneumoniae* DNA in buffy-coat samples of 18 (20%) patients and 8 (9%) control subjects ($p = 0.03$). The matched odds ratio of association between AAA and the presence of *C. pneumoniae* DNA in buffy-coat samples was 2.6 (95%CI, 1.1-6.3).

Utilizing the MIF test, specific IgG antibodies to *C. pneumoniae* were detected in 85 (97%) patients and 71 (81%) control subjects. The matched odds ratio of association between AAA and seropositivity for *C. pneumoniae* was 6.8 (95% CI, 2-24). The association was stronger in the lower IgG titers than in the higher IgG titers (table 2). The GMT of IgG antibodies to *C. pneumoniae* was significantly higher in patients than in control subjects 1:191 and 1:97, respectively. A multivariate analysis was performed to adjust for classic risk factors of atherosclerosis, including hypertension, hypercholesterolemia, diabetes mellitus and pack-years of cigarette smoking. History of cerebrovascular disease, peripheral vascular disease and myocardial infarction was also included in this analysis. Odds ratios did not decrease after correction for these factors. Adjusted odds ratios were 2.9 (95% CI, 1-8.5) for circulating *C. pneumoniae* DNA, and 7.2 (95% CI, 1.7-31) for IgG antibodies to *C. pneumoniae*.

Table 2. Pairwise distribution of PCR results and IgG antibodies to *C. pneumoniae* in patients and control subjects

	Patient & control both positive	Patient only positive	Control only positive	Patient & control both negative	Matched odds ratio (95% CI)
PCR	2	16	6	64	2.6 (1.1-6.3)
IgG					
≥1:16	69	16	2	1	6.8 (2.0-24)
≥1:32	64	18	5	1	3.6 (1.2-12.4)
≥1:64	56	23	5	4	4.6 (1.7-15.5)
≥1:128	38	29	10	11	2.9 (1.4-6.7)
≥1:256	21	31	11	25	2.3 (1.4-6.2)
≥1:512	6	17	16	49	1.1 (0.5-2.3)

In one control subject in whom the IgG antibody titer was negative, *C. pneumoniae* DNA was detected in the buffy-coat sample by PCR. Detection of *C. pneumoniae* was not associated with a higher GMT. The GMT of IgG antibodies in the 26 cases with positive PCR was similar to the GMT in the 150 cases with negative PCR (1:128).

Table 3 shows the association between detection of circulating *C. pneumoniae* DNA and the prevalence of circulating IgG antibodies to *C. pneumoniae* in the study subjects and in four previous studies.

No evidence of association was found between smoking and either IgG antibody titers or the detection of *C. pneumoniae* DNA in buffy-coat samples. The pack-years of cigarette smoking

were 20 (±17) in subjects who were PCR positive versus 30 (±29) in subjects who were PCR negative (P = 0.1). The pack-years of cigarette smoking were 30 (±29) and 18 (±14) in subjects with positive IgG titers and negative IgG titers, respectively (p = NS).

No association was found between age and detection of circulating *C. pneumoniae* DNA. The mean age of PCR-positive and PCR-negative subjects was 67 (±10) years and 67 (±7) years, respectively (P = NS). Results of serological investigations were similar: the mean age of IgG-positive and IgG-negative subjects was 66 years (±10) and 67 years (±7), respectively (P = NS).

A multivariate analysis to control for classic risk factors of atherosclerosis showed that neither PCR-positive nor IgG-positive results in control subjects were associated with those risk factors.

Discussion

This study shows an association between AAA and *C. pneumoniae*, which was demonstrated by a significantly higher detection rate of *C. pneumoniae* DNA in buffy-coat samples of patients with AAA than in samples of control subjects. The serological results emphasize the association between AAA and *C. pneumoniae*. Patients had IgG antibodies to *C. pneumoniae* more frequently and at higher titers than control subjects. The odds ratios did not decrease after adjustment for other risk factors of abdominal aneurysms, which implies that chlamydial infection is an independent risk factor for AAA. To minimize bias and confounding factors, we matched patients and control subjects for sex and age. In addition, to prevent differences in socioeconomic status, friends and neighbors of patients were included as control subjects, when possible.

The seroprevalence of *C. pneumoniae* was high, even in the control group. These results are consistent with the seropositivity rates reported previously among healthy population [19-21].

Eight pathological studies investigated the presence of *C. pneumoniae* in AAA, but only three included control tissue samples (table 4). Two studies failed to detect *C. pneumoniae* DNA in AAA [22, 23], and in one study PCR was not performed [24]. The remaining five studies reported rates of positive PCR results ranging from 35 to 100% [3, 13, 25-27].

It has been suggested that detection of circulating *C. pneumoniae* DNA might be an appropriate method to identify patients with chronic *C. pneumoniae* infection [13, 14]. There is, however, variation between published reports in the detection rates of *C. pneumoniae* DNA (tables 3, 4). These differences might be partly explained by population differences and methodological differences, including the DNA extraction method and the PCR assay [28]. Another important difference is the type of material tested. In three studies peripheral blood mononuclear cells were tested [13, 14, 29], in one study sera samples [30], and in the present study buffy-coat samples. As stated by Boman and Gaydos [28], it remains to be determined which sample type (buffy-coat, peripheral blood mononuclear cells or monocytes) is the most appropriate and provides the best results. The advantage of detection of *C. pneumoniae* DNA in buffy-coat samples is that the procedure of cell separation is easy and not time-consuming.

Table 3. Association between circulating *C. pneumoniae* DNA (PCR+) and IgG antibodies to *C. pneumoniae* (MIF+) in the present study and in four previous studies

Reference	Material	No. of specimens	PCR+, MIF+	PCR-, MIF-	PCR+, MIF-	PCR-, MIF+	*P* value
[13]	PBMC	41	19	15	0	7	<0.001
[14]	PBMC	1180	76	323	23	758	0.2
[29]	PBMC	153	81	8	3	61	0.07
[30]	serum	247	35	116	20	76	0.002
Present study	Buffy-coats	176	25	19	1	131	0.3

MIF, microimmunofluorescence; PBMC, peripheral blood mononuclear cells.

Blasi et al. [13] have shown that there is a correlation between the presence *C. pneumoniae* DNA in peripheral blood mononuclear cells and its presence in the aneurysm wall. They investigated AAA tissue and peripheral blood mononuclear cells of 41 patients with AAA, and in 39% of the patients *C. pneumoniae* DNA was detected in both peripheral blood mononuclear cells and AAA tissue. However, no materials from control subjects were included in that study. The present study is the first case-control study that investigates the association between AAA and the detection of *C. pneumoniae* DNA in buffy-coat samples. Our results support the hypothesis that *C. pneumoniae* is associated with AAA.

Controversial results have been reported about the association between the circulating *C. pneumoniae* DNA and antibody titers to *C. pneumoniae* (table 3). Two previous studies have demonstrated an association between circulating *C. pneumoniae* DNA and circulating IgG antibodies to *C. pneumoniae* [13, 30]. Two other studies failed to demonstrate such an association [14, 29]. Moreover, in the present study no association was found between the presence of *C. pneumoniae* DNA in the buffy-coat samples and the prevalence of IgG antibodies.

Association between smoking and antibody titers to *C. pneumoniae* has been reported, and smoking was suggested to be a confounder of the association between seropositivity and atherosclerosis [31]. This finding was not confirmed by a recent study [32]. We found no association between cigarette smoking and either seropositivity or the presence of *C. pneumoniae* DNA in buffy-coat samples of patients with AAA. This suggests that smoking has no confounding effect on the association between *C. pneumoniae* and AAA.

Table 4. Studies on the presence of *C. pneumoniae* in abdominal aortic aneurysm specimens and control specimens

Reference	Number of specimens		No. (%) of specimens positive for *C. pneumoniae*			
	AAA	controls				
			PCR		IHC	
			AAA	Controls	AAA	Controls
[3]	12	9 (aorta tissue)	6/6 (100)		12/12 (100)	0/9 (0)
[13]	41	0[a]	17/41 (41)			
[22]	20	0[a]	0/20 (0)			
[23]	19	0[a]	0/19 (0)		19/19 (100)	
[24]	11	0[a]			6/11 (55)	
[25]	40	40 (aorta tissue)	14/40 (35)	2/40 (5)		
[26]	51	0[a]	26/51 (51)			
[27]	25	6 (vascular tissue)	11/25 (44)	3/6 (50)		

[a]No controls were included in the study; PCR, polymerase chain reaction; IHC, immunohistochemistry.

The pathogenesis of AAA is unknown. There is some evidence that genetic factors, familial clustering and several proteolytic factors are involved in the pathogenesis [2, 33]. *C. pneumoniae* may directly cause endothelial injury. It also might induce a chronic immunological activation, causing chronic endothelial cell damage and mediating a proteolytic process in the wall of the abdominal aorta [3, 34]. Similarly, it has been suggested that chlamydial lipopolysaccharide and chlamydial heat-shock protein 60 may promote atherogenesis by inducing mononuclear phagocyte foam cell formation and mononuclear phagocyte oxidation of low-density lipoprotein, respectively [35].

In conclusion, the results of our study indicate that *C. pneumoniae* is associated with AAA. Although a causal relationship was not established, our results provide additional evidence that the role of *C. pneumoniae* needs to be explored further for better understanding of the pathogenesis of AAA.

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Chapter 4

Impact of serological methodology on assessment of the link between *Chlamydia pneumoniae* and vascular diseases

Boulos Maraha, Martin den Heijer, Jan Kluytmans, Marcel Peeters

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Abstract

We assessed the impact of five serologic tests on the link between *Chlamydia pneumoniae* and abdominal aortic aneurysm (AAA). The results of the tests were inconsistent. Agreement among the five tests was generally poor. Detection of the link between *C. pneumoniae* and AAA depends on the serologic methodology chosen.

Introduction

Seroepidemiologic studies that had investigated the link between *Chlamydia pneumoniae* and vascular diseases reported inconsistent results, ranging from a strong link to no link at all [3]. This discrepancy might be due to methodological factors [15]. Two recent studies have demonstrated that the detection of a link between *C. pneumoniae* and coronary artery disease depends on the choice of serologic methods [6, 15]. The serologic link between *C. pneumoniae* and vascular diseases has been assessed by microimmunofluorescence (MIF) tests and enzyme-linked immunosorbent assays (ELISA). However, these serologic tests lack sufficient reliability and standardization [4]. A poor agreement among the results of these tests has been reported [6, 13, 15].

In this case-control study, we investigated whether the choice of serologic tests influences the detection of a link between *C. pneumoniae* and abdominal aortic aneurysm (AAA). Moreover, we evaluated the agreement among the results of these tests.

Materials and Methods, Results and Discussion

The study population was previously described [9]. Patients with AAA and healthy controls were included and matched by age and sex. Serum samples were tested for the presence of *C. pneumoniae* IgG antibodies by five serologic tests, i.e., *Chlamydia* MIF IgG (MRL Diagnostic), *Chlamydia* IgG SeroFIA (Savyon Diagnostics Ltd; Savyon-MIF), *Chlamydia* IgG rELISA Medac (Medac Diagnostica), SeroCP IgG (Savyon Diagnostics Ltd; Savyon-ELISA), Elegance *Chlamydia pneumoniae* IgG ELISA (Bioclone).

The MRL-MIF uses, as the antigen, purified *C. pneumoniae* (strain TW 183) elementary bodies (EB) diluted in 3% yolk sac to add contrast to the background. According to the manufacturer's product information, the EBs are purified by removing the genus-specific lipopolysaccharide (LPS). The Savyon-MIF and the Savyon-ELISA also use purified *C. pneumoniae* (strain TW 183) EB as the antigen. The Medac-rELISA uses a recombinant LPS-fragment as the antigen. For the Bioclone-ELISA, purified *C. pneumoniae* outer membrane protein complexes are used as the antigen. All tests were performed and interpreted in a blinded fashion by the same technician, according to the manufacturers' instructions.

We used odds ratios and a 95% confidence interval for estimating the relative risk. Kappa (κ) values were used to assess the agreement among the tests. The following guidelines were used in the interpretation of κ: if κ was <0.2, agreement was poor, if κ was 0.21 to 0.4, agreement was fair, if κ was 0.41 to 0.6, agreement was moderate, if κ was 0.61 to 0.8,

agreement was good, and if κ was 0.81 to 1.0, agreement was very good [1]. *P* values of <0.05 were considered statistically significant.

The study population included 88 patients with AAA and 88 healthy controls. The characteristics of the study population are shown in table 1. The results of the five tests were inconsistent (table 2). In the patient group, seropositivity rates varied from 52% (46 of 88) with the Medac-rELISA to 97% (85 of 88) with the MRL-MIF. In the healthy controls, a similar variation was found: 55% (48 of 88) were positive with the Medac-rELISA compared to 97% (85 of 88) with the Bioclone-ELISA.

Table 1. Baseline characteristics of patients with AAA and control subjects[a]

Characteristic	No. of AAA patients	No. of control subjects	*P* value
Age (yr) (range)	69 (45-85)	67 (44-83)	NS [c]
Male/female	81/7	81/7	NS
Aneurysm			
Under control	35		
Operated	53		
Elective	40		
Symptomatic	7		
Ruptured	6		
Family history of aneurysm	16	9	NS
History			
Myocardial infarction	26	9	<0.01
Cerebrovascular disease	9	4	NS
Pulmonary embolism	2	3	NS
Chronic obstructive pulmonary disease	24	13	NS
Peripheral vascular disease	19	4	<0.01
Diabetes mellitus	6	4	NS
Smoking			
Pack-yr of cigarette smoking[b] (±SD)	34 (±33)	23 (±22)	<0.01
Medication			
Antihypertensive drugs	48	28	<0.01
Cholesterol-lowering drugs	28	8	<0.001
Nonsteroidal anti-inflammatory drugs	37	17	<0.01
Mean systolic blood pressure, mmHg (SEM)	159 (2.4)	158 (2.6)	NS
Mean diastolic blood pressure, mmHg (SEM)	85 (1.2)	87 (1.2)	NS
Mean cholesterol, mmol/l (SEM)	5.7 (1.13)	5.7 (0.10)	NS

[a] Patients (*n* = 88) and controls (*n* = 88) were matched by age and sex; [b] A pack-year was defined as smoking 20 cigarettes/day for 1 year; [c] NS, not significant.

The MRL-MIF was the only test that demonstrated a significant link between *C. pneumoniae* and AAA. The other four tests failed to demonstrate any link. However, the MRL-MIF also failed to demonstrate a link when higher IgG titers were used as cutoffs.

Although the results of the two MIF used in our study were read by the same technician, there was poor agreement between the two tests. This implies that in addition to the subjective reading of MIF results, other factors may contribute to disagreement among results of *C. pneumoniae* serologic tests. The test procedure, the type of antigen, the antigen's purity, and the concentration of the antigen may also account for poor agreement among the results of these serologic tests [5]. The link between low titers of *C. pneumoniae* IgG and AAA, demonstrated by the MRL-MIF, might be the result of a cross-reaction to the antigen used in the test from sources other than *C. pneumoniae* [8]. These sources, either infectious or noninfectious, might be associated with AAA and confound the association between *C. pneumoniae* and AAA.

Table 2. Association between *C. pneumoniae* seropositivity and AAA according to five different tests[a]

Test	IgG titer or index value	No. of AAA patients	No. of control subjects	Odds ratio (95% confidence interval)
MRL-MIF	- IgG≥ 1:16	85	71	6.8 (2.0-24.1)
	- IgG≥ 1:512	24	21	1.2 (0.6-1.8)
Savyon-MIF	- IgG≥ 1:64	79	79	1.0 (0.4-2.7)
	- IgG≥ 1:512	21	20	0.9 (0.5-1.8)
Medac-rELISA	- IgG≥ 1:100	46	48	0.9 (0.5-1.7)
	- IgG≥ 1:400	23	18	1.4 (0.7-2.8)
Savyon-ELISA[b]	- index> 1.10	67	71	0.8 (0.4-1.6)
Bioclone-ELISA[c]	- index> 1.10	84	85	0.7 (0.2-3.4)
	- index >3.0	20	16	1.3 (0.6-2.8)

[a] Eighty-eight patients and 88 controls were tested. [b] For the Savyon-ELISA, high titers were not found. [c] According to test kit instructions, an index value of >3 corresponds to an IgG titer of > 1:512.

The agreement among the results obtained by the five serologic tests was generally poor (table 3). Inter- and intralaboratory variations and a poor agreement among results of serologic tests of *C. pneumoniae* have also been demonstrated by others [6, 13, 15]. Ranges of agreement from 59% to 90% have been reported [13, 15]. Hoymans et al. [6] found poor agreement between results of the MIF and the Medac-rELISA, but three other ELISA showed moderate to good agreement in results with the MIF [6].

There is evidence that *C. pneumoniae* serologic tests are less specific than previously realized [5, 8, 11]. Cross-reactivity between *C. pneumoniae* and *Chlamydia* species in the MIF has been demonstrated [8, 11]. This is probably due to a lack of LPS removal from the EB during antigen preparation [11]. It is also possible that *Chlamydia*-like microorganisms,

Bordetella pertusis and parvovirus cause serologic antigenic cross-reactivity with *C. pneumoniae* [6, 7, 10, 12, 14].

Our results support the findings of recent studies which have shown that methodology has an important impact on whether a link is found between *C. pneumoniae* and vascular diseases [6, 15]. This indicates that methodological factors are partly responsible for the conflicting results in the literature concerning the role of *C. pneumoniae* in the development of vascular diseases [2, 3].

This study shows that the detection of a serologic link between *C. pneumoniae* and AAA depends on which test is used to measure *C. pneumoniae* antibodies. Further studies should focus on optimizing and standardizing *C. pneumoniae* serologic methods.

Table 3. Agreement of κ values between the serological results of the five different tests for the patient group and the healthy controls

			κ value[a]			
Group	Test	IgG titer or index value	Savyon-MIF	Medac-rELISA	Savyon-ELISA	Bioclone-ELISA[b]
Patients	MRL-MIF	IgG≥ 1:16	0.12	0.02	0.14	0.55
	Savyon-MIF	IgG≥ 1:64		0.12	0.45	0.42
	Medac-rELISA	IgG≥ 1:100			0.09	0.05
	Savyon-ELISA	index>1.10				0.26
Controls	MRL-MIF	IgG≥ 1:16	0.11	0.06	0.12	0.25
	Savyon-MIF	IgG≥ 1:64		0.09	0.55	0.47
	Medac-rELISA	IgG≥ 1:100			0.15	0.08
	Savyon-ELISA	index>1.10				0.25

[a] κ expresses the agreement between the tests regarding nominal scale variables (positive and negative results). [b] The Bioclone-ELISA had an index value of >1.10.

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Chapter 5

Chlamydia pneumoniae, systemic inflammation and the risk of venous thrombosis

Boulos Maraha, Marcel Peeters, Benien van Aken, Martin den Heijer

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Abstract

Inflammatory mediators are involved in activation of the coagulation system, and elevated plasma concentrations of IL-6 and IL-8 are associated with an increased risk of venous thrombosis. Using serologic and molecular biologic tests, we investigated in a case-control study on patients with recurrent venous thrombosis the association between *Chlamydia pneumoniae* and venous thrombosis and we evaluated the relation between *C. pneumoniae* serology and the cytokines IL-6 and IL-8. The presence of *C. pneumoniae* antibody titers ≥ 1:16 was not associated with an increased risk of venous thrombosis (odds ratio 0.8 95% CI, 0.4-1.7). Circulating *C. pneumoniae* DNA was detected in only one patient and two control subjects. IgG antibody titers against *C. pneumoniae* were not correlated with the concentrations of IL-6 and IL-8. These results indicate that the inflammatory process shown in patients with venous thrombosis is not related to *C. pneumoniae*.

Introduction

Venous thrombosis is a multicausal disease and several genetic and acquired risk factors have been identified [5, 13]. Recently we have shown that elevated plasma concentrations of IL-6 and IL-8 are associated with an increased risk of venous thrombosis [14]. However, the cause of these elevated cytokine concentrations remains unknown.

Infections with *Chlamydia pneumoniae* have drawn many attention as risk factor for cardiovascular disease. It has been demonstrated that acellular components of *C. pneumoniae* stimulate the production of cytokines in blood mononuclear cells and induce cytokines production [6, 11]. So, it can be hypothesized that infection with *C. pneumoniae* is also a risk factor for venous thrombosis and that explains the elevated cytokine levels in patients with venous thrombosis.

Recently, two controversial reports have been published on the association between venous thrombosis and the serology of *C. pneumoniae*. Lozinguez et al. [8], reported IgG antibodies against *C. pneumoniae* a risk factor of venous thromboembolism using microimmunofluorescence (MIF) test. This finding was not supported by Koster et al. [7], who found no increased risk of deep-vein thrombosis in relation with elevated antibody titers against *C. pneumoniae*. However, they did not use the reference serologic method (MIF test) to measure antibodies against *C. pneumoniae*. Therefore, we questioned whether their results are a reliable parameter of *C. pneumoniae* infection [9].

In the current study, we investigated, in a case-control study, whether *C. pneumoniae* is associated with an increased risk for venous thrombosis, using serologic and molecular biologic tests. Furthermore, we investigated the relationship between *C. pneumoniae* serology and the concentrations of the cytokines IL-6 and IL-8.

Materials and Methods

The study population has been previously described in detail [2]. Briefly, 473 patients with two or more episodes of venous thrombosis from the files of the anticoagulant clinic of The Hague, The Netherlands, were approached, and 185 participated. The healthy control group was selected through a general practice in The Hague. From the 2812 approached subjects 532 subjects were ready to participate in the study, and the first 220 formed the control group. Specimens were collected throughout the year.

***C. pneumoniae* IgG antibodies**

IgG antibodies against *C. pneumoniae* (TW 183), in the sera of patients and controls, were measured using a MIF test (MRL, Cypress, Ca, USA) as described previously [3, 12]. Samples were tested at a dilution of 1:16. Positive samples were tested at a dilution of 1:32, 1:64, 1:128, 1:256, and 1:512. Samples were coded and the code number was revealed following the completion of all laboratory tests. All serologic tests were performed blind by the same technician. An IgG antibody titer $\geq$ 1:16 was considered as positive serology.

Polymerase chain reaction (PCR)

Buffy-coat samples were prepared by centrifuging EDTA-whole blood 3300 gX for 10 min. PCR samples were prepared by extracting *C. pneumoniae* DNA from 200 µL of the buffy-coat fraction using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Detection of *C. pneumoniae* DNA was performed using a validated PCR assay as described previously [4]. The primers CpnA (5'-TGA CAA CTG TAG AAA TAC AGC-3') and CpnB (5'-CGC CTC TCT CCT ATA AAT-3') were used in a PCR assay based on the sequence of the 16S rRNA gene. A negative control, containing all PCR reagents without specimens, was processed with every 5 samples. To control for inhibition of the PCR reaction, a second sample of each buffy-coat sample was spiked with *C. pneumoniae* target DNA. If the spiked sample was negative the sample was considered inhibited. Inhibited PCR samples were repurified and retested using 2.5 µL of specimen. To minimize the risk of contamination, sample processing and the PCR assay were performed in separate rooms, and to prevent carryover of previous PCRs UDG/dUTP were used. PCR products were visualized after electrophoresis in 2% ethidium bromide- stained agarose gels. To confirm positive results, 3 µL of the PCR product was spotted and hybridized with a 21 bp 5'-biotinylated probe Cpneu-B (5'-GAC ACA CGT ACA ATG GTT-3'). Hybridization signals were visualized using streptavidin-peroxidase and enhanced-chemiluminescence (ECL) detection reagents (Amersham, Little Chalfont, United Kingdom).

Inflammatory mediators

As described previously [14], plasma levels of IL-6 and IL-8 were measured using a commercial ELISA (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service CLB, Amsterdam, the Netherlands). The ELISA tests were performed blind. The detection limit for the ELISA was 1.2 pg/ml for IL-6 and 2.0 pg/ml for IL-8. Samples below the detection limit were designated as 0 pg/ml.

Statistical analysis

Odds ratios and 95% confidence interval were calculated as an estimate of the relative risk for recurrent venous thrombosis by means of logistic regression analysis and are adjusted for age and sex.

Results

The mean age of the patients and the control subjects was 61 years (range 23 to 88) and 51 years (range 21 to 84), respectively. The cases group consisted of 94 males and 91 females, and the control group 94 males and 126 females.

Serum for *C. pneumoniae* serology was available from 184 cases and 218 control subjects. Plasma concentration of IL-6 was available from 181 cases and 163 control subjects. Plasma concentration of IL-8 was available from 168 cases and 148 control subjects. Because samples were initially collected to evaluate the association between venous thrombosis and hyperhomocysteinemia and many assays were performed in that study [2], some samples were used up and were not more available for all assays in the present study.

The prevalence of seropositivity to *C. pneumoniae* was quite similar in patients and control subjects 91% and 92%, respectively. Table 1 shows the odds ratio at different cut-off for the IgG titer to *C. pneumoniae*. The odds ratio (adjusted for age and sex) for positive IgG titer ($\geq$ 1:16) was 0.8 (95% CI, 0.4-1.7). This indicates that antibody titer against *C. pneumoniae* is not a risk factor for venous thrombosis.

Table 1. *Chlamydia pneumoniae* IgG antibody titers and the risk of venous thrombosis

IgG titer	No. of case subjects	No. of control subjects	Odds Ratio (95% CI) (adjusted for age and sex)
Negative	17	18	1*
$\geq$ 1:16	167	200	0.8 (0.4 – 1.7)
$\geq$ 1:32	149	185	0.8 (0.5 – 1.4)
$\geq$ 1:64	139	174	0.9 (0.5 – 1.5)
$\geq$ 1:128	118	141	1.1 (0,7 – 1.7)
$\geq$ 1:256	72	95	0.8 (0.5 – 1.2)
$\geq$ 1:512	40	63	0.6 (0.4 – 1.0)

* Reference category.

Figures 1-4 show the correlation between the IgG titers and the concentrations of IL-6 and IL-8. There was no clear difference in IL-6 and IL-8 levels in subjects with or without positive *C. pneumoniae* titer. However, a few subjects with high levels of IL-6 or IL-8 (>20 pg/ml) were all seropositive (9 and 5, respectively).

Circulating *C. pneumoniae* DNA was detected by PCR in one patient (0.5%) and two control subjects (0.9%).

Figure 1. The relation between IL-6 concentration and IgG antibody titer against *Chlamydia pneumoniae* in control subjects.

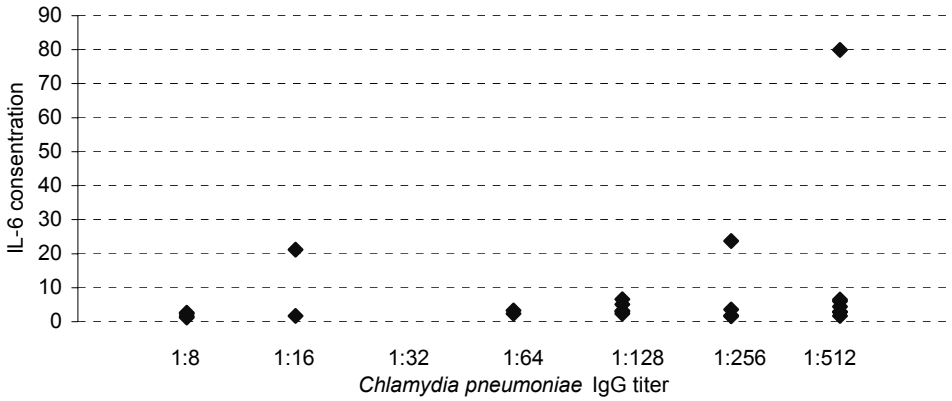


Figure 2. The relation between IL-8 concentration and IgG antibody titer against *Chlamydia pneumoniae* in control subjects.

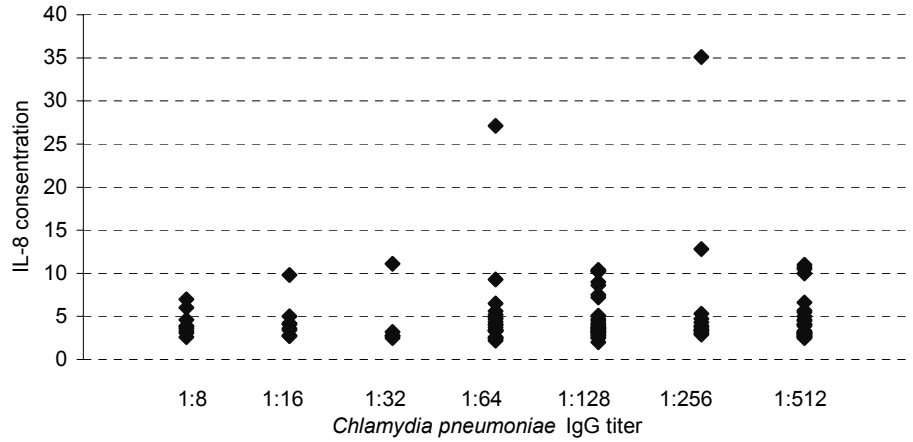


Figure 3. The relation between IL-6 concentration and IgG antibody titer against *Chlamydia pneumoniae* in patients with recurrent venous thrombosis.

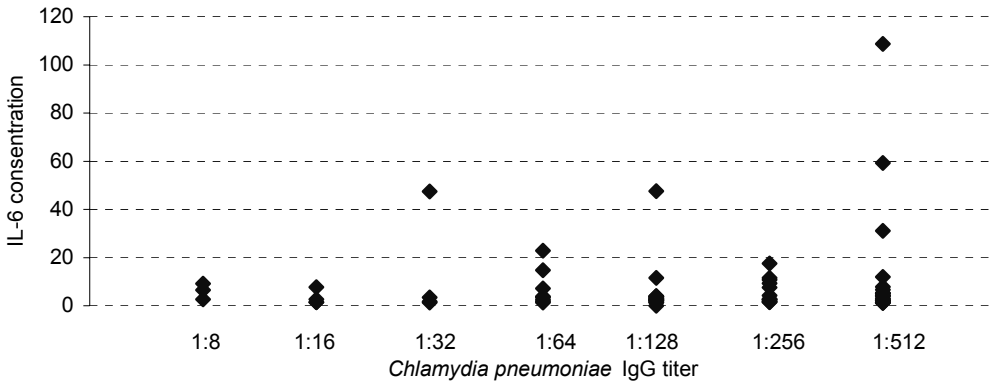
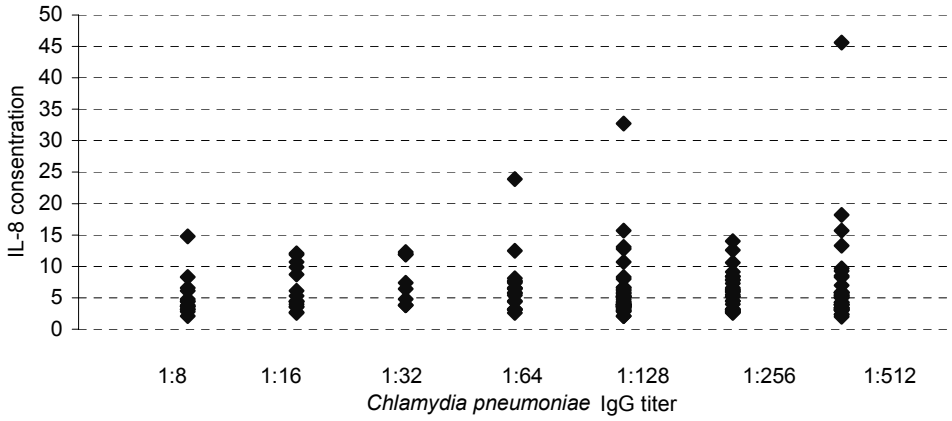


Figure 4. The relation between IL-8 concentration and IgG antibody titer against *Chlamydia pneumoniae* in patients with recurrent venous thrombosis.



Discussion

The results of the present study show that *C. pneumoniae* as detected by serology or PCR of peripheral blood cells is not associated with increased risk of venous thrombosis. Furthermore, IgG antibody titers against *C. pneumoniae* were in general not correlated with the concentrations of IL-6 and IL-8, although high levels of these cytokines were especially found in subjects with positive serology for *C. pneumoniae*. These results indicate that the inflammatory process shown in patients with venous thrombosis is not related to *C. pneumoniae* infection.

The studies of Lozinguez et al. [8] and Koster et al. [7] produced controversial results. Lozinguez et al. [8] demonstrated that *C. pneumoniae* seropositivity is correlated with an increased risk for deep-vein thrombosis. Koster et al. [7] did not find any association between *C. pneumoniae* serologic status and venous thrombosis. The negative results might be

explained by the fact that Koster et al. have used an Elisa test for antibody detection, which is not the standard method [9]. However, in the present study using the standard MIF test to measure IgG antibodies to *C. pneumoniae*, we didn't find any association too.

Respiratory infection with *C. pneumoniae* occurs more frequently in winter. Our study was performed throughout the year. Although the patients where enrolled especially during winter and the control subjects in autumn, one would expect higher prevalence in the patients group, which was not found. This implies that the findings of the present study were not biased by a season effect. Moreover, the control group is recruited from the general population in the same area as the patient group, so we think it is an appropriate control group.

Next to serology, we studied the detection of *C. pneumoniae* DNA in peripheral blood cells of patients with venous thrombosis, which has not been reported before. The detection rate of *C. pneumoniae* DNA was very low in patients with recurrent venous thrombosis as well as in control subjects. In a previous study [10], using the same PCR assay, we detected *C. pneumoniae* DNA in peripheral blood cells of 9% (8/88) of the healthy control subjects. An explanation for the difference in the detection rate between the present study and our previous study might be that the current study population is somewhat younger compared to the control subjects which were age-matched to patients with abdominal aneurysm. Furthermore, the reproducibility of *C. pneumoniae* PCR assays might be less than generally believed. A recent multicenter study showed major inter-laboratory differences in detection rate of *C. pneumoniae* in endarterectomy specimens [1].

Our data show that *C. pneumoniae* is not associated with an increased risk for venous thrombosis and show that *C. pneumoniae* does not explain the chronic inflammation associated with venous thrombosis. Thrombosis should be considered as a possible cause of the increase of cytokines in patients with venous thrombosis. This emphasizes the need for further studies to assess the mechanisms by which inflammatory markers are elevated in venous thrombosis.

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Chapter 6

Correlation between detection methods of *Chlamydia pneumoniae* in atherosclerotic and non-atherosclerotic tissues

Boulos Maraha, Hans Berg, Gert-Jan Scheffer, Anneke van der Zee, Anneke Bergmans, Jo Miseré, Jan Kluytmans, Marcel Peeters

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Abstract

Polymerase chain reaction (PCR) and immunohistochemistry (IHC) have been used to detect *Chlamydia pneumoniae* in vascular tissues. Discrepancies between the results of these two methods have frequently been reported. However, the correlation between PCR and IHC has not been analyzed yet. This study assesses the correlation between the detection of *C. pneumoniae* by PCR and IHC in 45 atherosclerotic and 50 non-atherosclerotic tissue specimens. Also, the presence of *Mycoplasma pneumoniae* in these 95 specimens was investigated. Correlation was found between the detection of *C. pneumoniae* by PCR and IHC in the atherosclerotic tissues. Both tests were positive in 10 specimens and negative in 17 specimens ($p = 0.003$). There was no significant correlation between PCR and IHC in non-atherosclerotic specimens (p = ns). *M. pneumoniae* was detected, by PCR, in one atherosclerotic specimen. The results show correlation between PCR and IHC in the detection of *C. pneumoniae* in atherosclerotic tissues, emphasize the association between *C. pneumoniae* and atherosclerosis, and support the specificity of the association between *C. pneumoniae* and atherosclerosis.

Introduction

Using polymerase chain reaction (PCR) and immunohistochemistry (IHC), the presence of *Chlamydia pneumoniae* has been demonstrated in atherosclerotic tissues, but in control vascular tissues this pathogen has been found less frequently [28]. These findings indicate that *C. pneumoniae* may play a role in the pathogenesis of atherosclerosis.

In addition to *C. pneumoniae*, several micro-organisms have been postulated as possible risk factors for atherosclerosis [5, 27]. The association between atherosclerosis and both cytomegalovirus and *Helicobacter pylori* has been extensively studied [5]. However, till now only one report on the presence of *Mycoplasma pneumoniae* in vascular tissues has been generated [23].

The microimmunofluorescence test (MIF), PCR and IHC have been used to explore the association between *C. pneumoniae* and atherosclerosis. Discrepancies among the results obtained by these methods have frequently been found [9, 11, 15]. Nothing, however, is known about the correlation between the results of these detection methods.

The present study assesses the correlation between the detection of *C. pneumoniae* by PCR and IHC. Also, the correlation between *C. pneumoniae* serology and the detection of this pathogen, by PCR and IHC, in vascular tissue specimens was analyzed. *M. pneumoniae*-PCR was performed to detect *M. pneumoniae* in atherosclerotic and non-atherosclerotic tissues.

Materials and methods

Specimens were collected from 95 patients undergoing cardiac surgery. Forty-five atherosclerotic specimens and 50 non-atherosclerotic tissue specimens (aortic-punchs) were collected. Each specimen was divided into two portions; one for PCR and one for IHC. The PCR portion was transported to the laboratory in a Tris-EDTA buffer containing 0.5% sodium dodecyl sulfate. In the laboratory, specimens were stored at -20^{o}C. The IHC portion was fixed in 10% phosphate-buffered formalin. In addition, sera were obtained from all patients to measure IgG antibodies to *C. pneumoniae*. Specimens were evaluated blind. Patients were not examined to assess the presence of *C. pneumoniae* clinical disease.

Polymerase chain reaction

DNA extraction from specimens was performed using the QIAamp DNA minikit (Qiagen, CA, USA). For the PCR template, 5 µL of 200 µL elution sample was used.

Detection of *C. pneumoniae* DNA was carried out by 16S rRNA gene-based PCR, using the primers CpnA: 5'-TGA CAA CTG TAG AAA TAC AGC-3' and CpnB: 5'-CGC CTC TCT CCT ATA AAT-3' as described previously [8].

Detection of *M. pneumoniae* was performed by PCR assay based on the P1 adhesin gene using the primers Pn1: 5'-GCC ACC CTC GGG GGC AGT CAG-3' and Pn2: 5'-GAG TCG GGA TTC CCC GCG GAG G-3' as described previously [10, 23].

To minimize the risk of contamination, strict PCR-anti-contamination precautions, such as the use of UDG/dUTP, were taken to prevent carry-over of previous PCR reactions. Sample processing and PCR assays were performed in separate rooms and a negative control was processed with every 5 samples. To control for inhibition of the PCR reaction, a second sample of each specimen was spiked with target DNA (*C. pneumoniae* or *M. pneumoniae*). Inhibited PCR samples were retested using 2.5 µl of specimen. PCR products were visualized after electrophoresis in 2% ethidium bromide-stained agarose gels.

To confirm positive results, 3 µl of the PCR product was spotted and hybridized, with the 21 bp 5'-biotinylated probe Cpneu-B:5' GAC ACA CGT ACA ATG GTT-3' in the case of *C. pneumoniae*-PCR and with the 5'-biotinylated probe MP2-B: 5'-GGT GAA GGA ATG ATA AGG CT-3' in the case of *M. pneumoniae*-PCR. Hybridization signals were visualized using streptavidin-peroxidase and ECL detection reagents (Amersham, UK).

Immunohistochemistry

Specimens were transported in 10% phosphate buffered formalin. Subsequently tissues were, in some cases after decalcification with EDTA, embedded in paraffin and sectioned at 5 µm. IHC staining was performed according to methods previously described [16]. Sections were stained with a mouse anti-*C. pneumoniae* (TW 183) monoclonal antibody (DAKO Diagnostics, Denmark). *C. pneumoniae*-infected HL cells and a tissue section stained with

normal mouse ascites were used as a positive and a negative control, respectively. Positive and negative controls were run with each batch of specimens.

Microimmunofluorescence test

C. pneumoniae (TW 183) IgG antibodies in the sera were determined using a MIF test (MRL Diagnostics, CA) as described previously [7, 24]. An IgG antibody titer $\geq$ 1:16 was considered positive. All serologic tests were performed blind by the same technician.

Statistical analysis

Correlation between the results of the different assays was analyzed by the Cohen's Kappa test.

Results

Atherosclerotic specimens were obtained from 38 males and 7 females (mean age 63 years, range 41-86). Non-atherosclerotic lesions were obtained from 41 males and 9 females (mean age 65 years, range 44-79).

Forty-five atherosclerotic tissue specimens and 50 non-atherosclerotic tissue specimens were available for the PCRs assays. Specimens for IHC assay were obtained from all patients, but 3 atherosclerotic specimens and 4 non-atherosclerotic specimens were missed. Blood samples for *C. pneumoniae* serology were available from 91 patients (41 in the atherosclerotic group and 50 in the non- atherosclerotic).

C. pneumoniae DNA was detected by PCR in 22% (10/45) of atherosclerotic specimens and in 10% (5/50) of non- atherosclerotic specimens. IHC staining was positive for *C. pneumoniae* in 60% (25/42) of the atherosclerotic specimens and in 9% (4/46) of the non-atherosclerotic specimens. The correlation between the detection of *C. pneumoniae* by PCR and IHC is shown in table 1. There was a correlation between the detection of *C. pneumoniae* by PCR and IHC in atherosclerotic specimens (p = 0.003). A poor correlation was found between the results obtained by PCR and IHC in non-atherosclerotic specimens (p = NS).

Table 1. Correlation between polymerase chain reaction (PCR) and immunohistochemistry (IHC) in the detection of *C. pneumoniae* in atherosclerotic and non-atherosclerotic tissue specimens

	atherosclerotic specimens		non-atherosclerotic specimens	
	IHC negative	IHC positive	IHC negative	IHC positive
PCR negative, no.	17	15	38	4
PCR positive, no.	0	10	4	0
Correlation, Kappa (p)	0.35 (0.003)		0.1 (ns)	

The MIF test detected *C. pneumoniae* IgG antibodies in 88% (36/41) of patients in the atherosclerotic group and in 96% (48/50) of patients in the non- atherosclerotic group. Table 2 demonstrates the correlation between *C. pneumoniae* serology and the results obtained by either PCR or IHC. There was no significant correlation between *C. pneumoniae* serology and both PCR and IHC.

M. pneumoniae was detected by PCR in one atherosclerotic tissue specimen. All non-atherosclerotic specimens were *M. pneumoniae*-negative.

Table 2. Correlation between serology (MIF), polymerase chain reaction (PCR) and immunohistochemistry (IHC)

Atherosclerotic specimens				
	PCR negative	PCR positive	IHC negative	IHC positive
MIF negative, no.	5	0	2	2
MIF positive, no.	26	10	12	22
Correlation, Kappa, (*p*)	0.1 (ns)		0.07 (ns)	
Non-atherosclerotic specimens				
	PCR negative	PCR positive	IHC negative	IHC positive
MIF negative, no.	1	1	2	0
MIF positive, no.	44	4	40	4
Correlation, Kappa, (*p*)	0.04 (ns)		0.01 (ns)	

Discussion

This study shows the following features: there was correlation between PCR and IHC in the detection of *C. pneumoniae* in atherosclerotic tissues, but a poor correlation was found in non-atherosclerotic specimens. The MIF test was not correlated with the results of PCR and IHC. *M. pneumoniae* was detected in one atherosclerotic specimen. All non-atherosclerotic specimens were *M. pneumoniae*-negative.

Several studies have reported a wide variation in the detection rate of *C. pneumoniae* between PCR and IHC [9, 15]. In general, more positive results are obtained with IHC than PCR. This was also the case in the present study. It has been suggested that these discrepancies might reflect differences in the sensitivity and specificity of these two methods [28]. Focal localization of *C. pneumoniae* in vascular tissues and the presence of components that inhibit PCR may influence the results of IHC and PCR [15]. However, discrepancy between detection rates of *C. pneumoniae* by PCR and IHC does not imply that there is no correlation between these methods. In this study, with regard to the atherosclerotic tissues there was correlation between the results of IHC and PCR, but no such correlation was found in non-atherosclerotic specimens. We can not explain why no correlation was found in non-atherosclerotic tissues. A poor correlation may be due to the relatively small number of specimens tested and to the low positive rate by PCR and IHC in the non-atherosclerotic

specimens. A possible explanation for this is that after initial infection, *C. pneumoniae* does not persist in non-atherosclerotic tissues [11].

We analyzed the correlation between PCR and IHC in 10 studies in which these methods were used to detect *C. pneumoniae* in arterial tissues [4, 6, 11, 12, 14, 17, 18, 19, 25, 26]. Correlation between PCR and IHC results was found in 7 studies (table 3). Our findings together with previous reports provide evidence that the results of PCR and IHC are correlated in atherosclerotic tissues. This finding emphasizes the association between *C. pneumoniae* and atherosclerosis, and provides an additional histological evidence that supports the hypothesis that *C. pneumoniae* might be involved in the pathogenesis of atherosclerosis.

Table 3. Correlation between polymerase chain reaction (PCR) and immunohistochemistry (IHC) in 10 studies in which arterial tissues were investigated

Reference	Material	No.	PCR+ [a] & IHC+	PCR- [b] & IHC-	PCR+ & IHC-	PCR- & IHC+	Correlation, Kappa (*p*)
[4]	Coronary atheromas	38	9	18	3	8	0.4 (0.01)
[11]	Coronary arteries	38	1	25	5	7	0.05 (0.8)
[17]	Coronary atheromas	30	8	14	5	3	0.45 (0.01)
[26]	Arterial atheromas	7	2	4	0	1	0.7 (0.05)
[19]	Peripheral arteries	17	2	9	0	6	0.3 (0.1)
[14]	AAA [c]	9	5	3	1	0	0.8 (0.02)
[12]	Carotid endarterectomy	16	3	8	0	5	0.4 (0.055)
[25]	Coronary arteries	12	3	5	2	2	0.3 (0.3)
[18]	Coronary arteries	49	2	41	1	5	0.3 (0.007)
[6]	Coronary arteries	60	12	38	2	8	0.6 (<0.001)

[a] + Positive; [b] – Negative; [c] AAA; abdominal aortic aneurysm.

In the present study no correlation was found between *C. pneumoniae* serology and the detection of *C. pneumoniae* either by PCR and IHC. Controversial reports have been published on the association between *C. pneumoniae* serology and the detection of this pathogen in vascular tissues [1]. We analyzed 7 studies in which *C. pneumoniae* IgG antibodies were determined and PCR was used to detect *C. pneumoniae* in arterial tissues [2, 3, 13, 17, 20-22]. Correlation between PCR and serological results was found in 3 studies (table 4).

Large differences in detection rate of *C. pneumoniae* between atherosclerotic lesions and non-atherosclerotic controls have been reported, 51% (303 of 597 specimens) and 4% (5 of 131 specimens), respectively [9]. This is consistent with the results of the present study, since *C. pneumoniae* was more frequently detected in atherosclerotic tissues than non-atherosclerotic tissues. It has been suggested that *C. pneumoniae* might be less frequently disseminated to non-atherosclerotic tissues or it does not persist for a long time in these tissues [11].

Table 4. Correlation between PCR and serology (MIF) in 7 studies in which arterial tissues were investigated

Reference	Material	No.	PCR+ [a] & MIF+	PCR - [b] & MIF-	PCR+ & MIF-	PCR- & MIF+	Correlation Kappa, (*p*)
[17]	Coronary atheromas	29	8	2	5	14	0.2 (0.1)
[21]	Coronary arteries	158	34	24	0	100	0.1 (0.005)
[2]	AAA[c]	51	25	9	1	16	0.3 (0.004)
[22]	Carotid endarterectomy & restenotic bypass	70	21	2	0	47	0.03 (0.3)
[20]	Carotid endarterectomy	47	7	8	0	32	0.07 (0.2)
[3]	AAA	41	16	14	1	10	0.3 (0.01)
[13]	Coronary atheromas	40	2	16	1	21	0.02 (0.7)

[a] + Positive; [b] - Negative; [c] AAA; abdominal aortic aneurysm.

In a previous study, we detected *M. pneumoniae* in one of 39 atherectomy specimens and in two of 64 degenerative heart valves [23]. In the present study, we simultaneously performed *C. pneumoniae*- and *M. pneumoniae*-PCR on atherosclerotic and non-atherosclerotic tissues. One atherosclerotic specimen was positive in the *M. pneumoniae*-PCR, and 15 specimens (10 atherosclerotic and 5 non-atherosclerotic) were positive in the *C. pneumoniae*-PCR. These results add a new evidence for the specificity of the association between *C. pneumoniae* and vascular disease.

In conclusion, the results of this study show a good correlation between PCR and IHC in the detection of *C. pneumoniae* in atherosclerotic tissues. This supports the association between *C. pneumoniae* and atherosclerosis and indicates that *C. pneumoniae* might be involved in the development of atherosclerosis. The very low detection rate of *M. pneumoniae* in atherosclerotic specimens supports the specificity of the association between *C. pneumoniae* and atherosclerosis.

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Chapter 7

Is *Mycoplasma pneumoniae* associated with vascular disease?

Boulos Maraha, Anneke vd Zee, Anneke Bergmans, Ma Ling Pan, Marcel Peeters, Hans Berg, Gert-Jan Scheffer, Jan Kluytmans

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Introduction

Several studies have postulated a link between infectious agents and atherosclerosis [3, 12]. Epidemiological and pathological studies have shown an association between *Chlamydia pneumoniae* and atherosclerosis [3, 12]. *C. pneumoniae* has been detected in vascular tissues and degenerative heart valves [3, 9]. It has been suggested that *Mycoplasma pneumoniae* might also play a role in the development of atherosclerosis [12]. Myocarditis, pericarditis, cerebral stroke, and vasculitis have been previously associated with *M. pneumoniae* infection [1, 4, 5, 11]. *M. pneumoniae* might be seen as a plausible candidate to play a role in the pathogenesis of atherosclerosis, because it has been related to cardiovascular disease and its ability to induce chronic inflammation [12]. Taylor-Robinson and Thomas have postulated that there is a need to investigate the presence of *M. pneumoniae* in vascular tissues, because there is a similarity in epidemiological behavior and antibiotic susceptibility between *M. pneumoniae* and *C. pneumoniae* [12]. Furthermore, *M. fermentans* has been detected in 1 of 19 human arterial specimens that were positive for *C. pneumoniae* [10], and it has been shown that *M. gallisepticum* exhibits tropism for arterial tissue in turkeys [2]. However, nothing is known about the presence of *M. pneumoniae* in human atherosclerotic vascular tissues. Therefore, we investigated the presence of *M. pneumoniae* in atherectomy specimens and in degenerative heart valve (DHV) specimens.

Materials and Methods, Results and Discussion

Atherectomy specimens (n = 39) and DHV specimens (n = 64) were obtained from patients undergoing cardiac surgery for coronary artery bypass graft surgery and heart valve replacement, respectively. Specimens were transported in a Tris-EDTA buffer containing 0.5% sodium dodecyl sulfate. DNA extraction was performed using a commercial kit (QIAamp DNA Minikit; Qiagen, Hilden, Germany). Detection of *M. pneumoniae* was performed by a PCR assay based on the P1 adhesin gene and using the primers Pn1 (5'-GCC ACC CTC GGG GGC AGT CAG-3') and Pn2 (5'-GAG TCG GGA TTC CCC GCG GAG G-3') as described previously [7]. PCR products were visualized after electrophoresis in 2% ethidium bromide-stained agarose gels (Fig. 1). To confirm the PCR results, the PCR product was spotted and hybridized with the 5'-biotinylated probe MP2-B (5'-GGT GAA GGA ATG ATA AGG CT-3'). Hybridization signals were visualized using streptavidin-peroxidase and enhanced chemiluminescence (ECL) detection reagents (Amersham, Little Chalfont, United Kingdom).

M. pneumoniae was detected in only 1 (2.5%) of the 39 atherectomy specimens and in 2 (3%) of the 64 DHV specimens. This is the first study on detection of *M. pneumoniae* in vascular tissues. Thus, there are no other data available to compare our results with.

In general, several factors can influence the results of PCR-based studies on detection of microorganisms in vascular tissues, including contamination, inhibition, size and composition of specimens, and the lack of standardized methods for DNA extraction and PCR assays [8].

We used a validated PCR assay with a high sensitivity (92 to 100%) [7]. Strict PCR anticontamination precautions were taken, such as the use of UDG/dUTP, to prevent carryover of previous PCRs. Sample processing and the PCR assay were performed in separate rooms. A negative control was processed with every set of five samples. To control for inhibition of the PCR, a second sample of each specimen was spiked with *M. pneumoniae* target DNA, and inhibited PCR samples were again purified and retested. Also, this PCR assay was used in our laboratory on respiratory samples. A total of 179 throat samples obtained from patients with respiratory tract infections were tested. These samples were sent to the laboratory for detection of respiratory pathogens. The *M. pneumoniae* PCR detected *M. pneumoniae* in 27 (16%) of the 179 samples. These results indicate that the PCR assay we used was sensitive and able to detect *M. pneumoniae*. However, it should be mentioned that there is no similarity in composition between vascular tissues and throat specimens.

It has been found in a serological study that, in contrast to *C. pneumoniae* antibodies, *M. pneumoniae* antibodies are not associated with recurrent events in patients with unstable angina [6]. Using PCR, we were unable to detect *M. pneumoniae* in the great majority of the 103 tested specimens. The results of this study do not support the hypothesis that *M. pneumoniae* is an important factor in the development of vascular disease. Further investigations are needed to confirm our results.

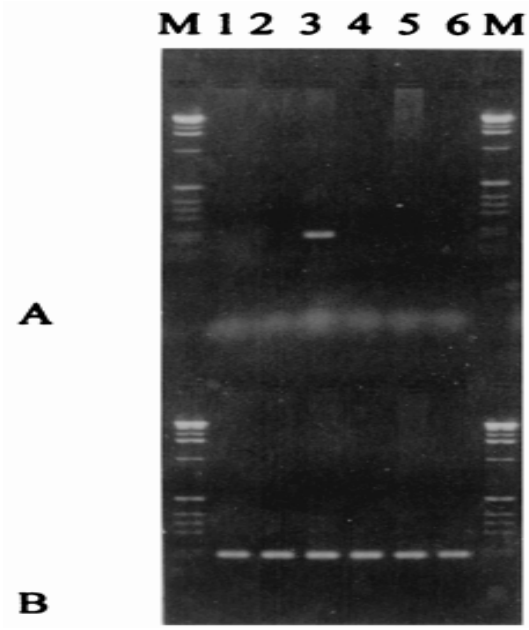

Figure 1. Agarose gel electrophoresis of PCR products obtained with P1 adhesin gene primers. (A) Lane M, 1-kb ladder; lanes 1 to 5, vascular tissues of five patients; lane 6, negative control. (B) Lanes 1 to 6 contain the same samples shown in panel A spiked with *M. pneumoniae* target DNA (positive controls).

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Chapter 8

Is the perceived association between *Chlamydia pneumoniae* and vascular diseases biased by methodology?

Boulos Maraha, Hans Berg, Marjolein Kerver, Steef Kranendonk, Jaap Hamming, Jan Kluytmans, Marcel Peeters, Anneke van der Zee

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Abstract

Inter- and intralaboratory inconsistency in detection rates of *Chlamydia pneumoniae* in vascular specimens has been demonstrated. In this study, 66 vascular tissue specimens from 66 patients with vascular disease were tested by three polymerase chain reaction (PCR) assays: a 16S PCR-based reverse line blot (RLB) assay, a single-step PCR, and a nested PCR. Also, we explored the impact of different DNA polymerase enzymes on the results based on gel electrophoresis and hybridization. The PCR results by gel electrophoresis in the single-step PCR depended on which DNA polymerase was used. All samples were negative with Amplitaq Gold DNA polymerase, and 54.5% (36/66) were positive with the conventional Taq DNA polymerase. All samples were negative after hybridization with a *C. pneumoniae*-specific probe. In the nested PCR all specimens were negative by gel electrophoresis and after hybridization. The RLB failed to detect *C. pneumoniae* in any specimen, however 20 specimens were *Chlamydia* species (spp.)-positive. The sequence analysis of six of these samples demonstrated *Chlamydia*-like organisms. RLB detected *Chlamydia* spp. DNA in water and in the elution buffer after passage of the Qiagen columns (11/40). This study identified factors that may influence the detection of *C. pneumoniae* DNA in vascular tissues, and consequently biasing the link between *C. pneumoniae* and vascular diseases. It is strongly recommend to use DNA polymerases that have to be activated, to decontaminate with dUTP/UNG, to hybridize with specific probes, to include sufficient controls, and to use molecular grade water.

Introduction

The association between *Chlamydia pneumoniae* and vascular diseases has gained considerable attention in recent years. Culturing of *C. pneumoniae* is difficult and only in sporadic cases the microorganism has been successfully isolated. Therefore, molecular detection methods have been widely used to investigate the association between *C. pneumoniae* and vascular diseases. Several polymerase chain reaction (PCR) assays have been described and those described by Gaydos et al. [17], Campbell et al. [11], and Tong and Sillis [50] have been most widely used in various investigations. PCR assays for the detection of *C. pneumoniae* are still not standardized and investigators in the field face many problems, including unspecific amplification, contamination, and poor sensitivity [9, 10, 15].

Major inter- and intralaboratory inconsistency in detection rates of *C. pneumoniae* in vascular specimens has been demonstrated recently [3]. The same study also showed that many false positive results were obtained. Several issues have been linked to the variation in detection rate of *C. pneumoniae*, such as specimen collection and processing, DNA-extraction, the choice of primers, visualization of PCR products, false positive and false negative signals [9].

In this study, we investigated vascular tissue specimens, from patients undergoing surgery, by a 16S PCR-based reverse line blot (RLB) assay to detect *C. pneumoniae* DNA as well as

Chlamydia species (spp.) DNA. In addition, we performed the PCR assays described by Gaydos et al. [17] and Tong and Sillis [50]. Also we evaluated the impact of two DNA polymerase enzyme types on the detection of *C. pneumoniae* in vascular tissues.

Materials and methods

Vascular tissue specimens were obtained from 66 patients with vascular disease and undergoing vascular surgery. Sixty-one patients were subjected to surgery because of peripheral atherosclerotic disease and 5 patients because of abdominal aortic aneurysm. The specimens included 40 atherosclerotic specimens from the femoral artery, 9 from the carotid artery, 7 from the iliac artery, 5 from the popliteal artery, and 5 abdominal aortic aneurysm specimens. Specimens were transported to the laboratory in a Tris-EDTA buffer containing 0.5% sodium dodecyl sulfate. In the laboratory, vascular specimens were stored at -70 ^{0}C until further processing. The local ethical committee approved the study and included patients gave their informed consent.

Polymerase chain reaction

DNA extraction from specimens was performed using the QIAamp DNA minikit (Qiagen, Hilden, Germany) as described previously [7]. Detection of *C. pneumoniae* DNA was carried out by the following PCR assays: assay-A1, a single-step *C. pneumoniae* PCR targeting the 16S rRNA gene essentially as described by Gaydos et al. [17]. Assay-A2, as assay-A1 with the following modifications: uracil-DNA glycosylase (UNG) was used prior to amplification and dUTP was used instead of dNTP's; AmpliTaqGold DNA polymerase was used instead of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.). Assay-B1, a nested *C. pneumoniae* PCR targeting the ompA gene essentially as described by Tong and Sillis [50]. Assay-B2, as assay-B1 but using AmpliTaqGold DNA polymerase instead of Taq DNA polymerase. Assay-C, an in-house *Chlamydia* spp. PCR targeting the 16S rRNA gene. Assay-D, a *Chlamydia* spp. PCR targeting the 16S rRNA gene as described by Ossewaarde and Meijer [41]. Assay-D was performed only for the identification by sequencing. The primers and probes used in assays-A to assay-D are listed in table 1.

To minimize the risk of contamination and to prevent carryover from previous PCR reactions dUTP and UNG were used in all assays except assay-A1 and the nested PCR (assay-B1 and assay-B2). Sample processing, preparation of PCR mixtures, and PCR assays were performed in separate rooms. In all PCR runs of each assay positive controls in dilution series were included to monitor sensitivity. This was accomplished by spiking 5 known concentrations of *C. pneumoniae* DNA (strain TW-183) (range, 0.01 IFU to 100 IFU) in a pool of negative clinical material. In assay-C, we used a clinical sample positive for *C. trachomatis* in the COBAS Amplicor PCR assay (Roche Diagnostics Systems Inc., Pleasanton, Calif.) as additional control for the *Chlamydia* genus-, the *C. pneumoniae*-, and the *C. trachomatis*-specific probes.

Negative controls were included after every four samples during processing and PCR. PCR's were performed in a PE 9600 Thermocycler (Perkin Elmer Cetus, Norwalk, Conn.) with the following settings:

Assay-A1 and -A2. The PCR reaction mixture contained 30 pmol of each primer, 3 mM $MgCl_2$, 200 µM of dNTP (assay-A1) or dUTP/UNG (assay-A2), 0.3 units of Taq DNA polymerase (assay-A1) or Amplitaq Gold DNA polymerase (assay-A2), and 5 µl extracted DNA. Only for assay-A2 0.25 units of UNG was used. The final reaction volume was 25 µl. The PCR program was as follows: 2 min at 50ºC, 10 min at 96ºC (only for assay-A2), followed by 40 cycles of 30s at 95ºC, 30s at 55ºC, and 1 min at 72ºC. A final step of 10 min at 72ºC completed the PCR**.**

Assays-B1 and -B2. The first PCR reaction mixture contained 10 pmol of each external primer, 1.5 mM $MgCl_2$, 200 µM of dUTP, 2.5 Units of Taq DNA polymerase (assay-B1) or Amplitaq Gold DNA polymerase (assay-B2), and 10 µl of extracted DNA in a final volume of 25 µl. The first PCR program consisted of 10 min at 96ºC, followed by 1 min 94ºC, 1 min 65ºC, and 1 min 72ºC. During 22 cycles the annealing temperature was lowered 1ºC every 2 cycles to 55ºC. Subsequently, 20 cycles were performed with 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC. The second PCR mixture contained 25 pmol of each internal primer, 3 mM $MgCl_2$, 200 µM of dUTP, 2.5 Units of Taq DNA polymerase (assay-B1) or Amplitaq Gold DNA polymerase (assay-B2), and 1 µl of the first PCR product in a final volume of 25 µl. The second PCR program consisted of 30 cycles of 94ºC for 1min, 50ºC for 1 min, and 72ºC for 1 min.

Assay-C. The PCR reaction mixture contained 20 pmol of primer CHLF, 10 pmol of primer CHLR-B, 12.5 µL Hotstar-MasterMix (Qiagen, Hilden, Germany). Five micro-liter of template DNA was added to the mixture and the final volume was 25 µl. Samples were subjected to the following PCR program: two cycles of 20 sec at 95ºC, 1 min at 66ºC and 1 min at 72ºC. Subsequently, every two cycles the annealing temperature was lowered by 2^{o}C to 56^{o}C, followed by 40 cycles of 20 sec at 96ºC, 1 min at 56ºC and 1 min at 72ºC. The PCR was completed with 7 min at 72ºC.

Assay-D: The PCR reaction contained 10 pmol of each primer, 12.5 µ of Hotstar-mastermix (Qiagen, Hilden, Germany). The PCR program was identical to that of assay-C described above.

Visualization of PCR products and interpretation of results

PCR products were visualized after electrophoresis in 2% ethidium bromide-stained agarose gels (MP agarose, Roche Biochemicals, Indianapolis, Ind). The PCR products (3 µl) of assay-A and assay-B were also spotted and hybridized with the appropriate 5'-biotin-labeled probe (table 1). Hybridization signals were visualized using streptavidin-peroxidase and ECL detection reagents (Amersham, UK).

PCR products of assay-C were obtained with a biotinylated primer. These products were hybridized to the *Chlamydia* spp.-, *C. pneumoniae*- and *C. trachomatis*-specific oligonucleotide probes that were covalently bound to a Biodyne C membrane (Amersham, UK) using a miniblotter (Immunetics, Cambridge, Mass.) in a RLB assay (table 1 and Figure 1). The technique of RLB hybridization has been previously described [47]. In brief; The PCR products were denaturated for 10 min at 99°C after dilution of 10 µl of the biotin-labeled PCR product in 150 µl of 2x SSPE–0.1% SDS. After denaturation, PCR products were added to the membrane and hybridized for 1 h at 42°C. Hybridization signals were visualized using streptavidin-peroxidase and ECL detection reagents (Amersham, UK).

PCR runs were judged by negative and positive controls. The results obtained by agarose gel electrophoresis were scored as either PCR-positive or PCR-negative. PCR samples that gave clear hybridization signals in dot spot or RLB analysis were scored *C. pneumoniae*-positive and/or *Chlamydia* spp.-positive, according to the probe used.

Statistical analysis

Statistical analysis was performed by SPSS for windows version 11.0. A $p < 0.05$ was considered statically significant.

Results

During surgery, vascular tissue specimens were obtained from 66 patients with vascular disease, 61 patients with peripheral atherosclerotic disease and 5 patients with abdominal aortic aneurysm. The mean age of patients was 61 years, range 40-89. Forty-nine patients (74.2%) were males and 17 (25.8%) were females.

Samples were subjected to PCR assays A1, A2, B1, B2, and C, and the sensitivity of each assay was experimentally monitored in each run by a dilution series of positive controls spiked in negative clinical material. The lowest detection limit was 0.1 IFU for the single-step PCR (assay-A1 and assay-A2) and the PCR-based RLB (assay-C). The nested PCR of assay-B1 and assay-B2 was less sensitive with 1.0 IFU as detection limit.

The results of the PCR assays are shown in table 2. In the single-step PCR, 36 specimens were PCR-positive when Taq Polymerase was used (assay-A1), and none of the specimens was PCR-positive when AmpliTaq Gold polymerase was used (assay-A2). However, none of the specimens that were PCR-positive in the gel electrophoresis could be confirmed after hybridization with a *C. pneumoniae*-specific probe.

In the nested PCR (assay-B1 and assay-B2) all specimens were PCR-negative and were also negative after hybridization.

The RLB (assay-C) failed to detect *C. pneumoniae* in any specimen, however 20 specimens were *Chlamydia* spp-positive (Figure 1). To further specify the *Chlamydia* spp. found, six of the samples were subjected to PCR assay-D and the 16S rRNA gene fragments obtained were sequenced. Using the BLAST server, the sequences obtained were compared with the

sequences in GenBank. The sequence analysis demonstrated that these strains were Chlamydia Research Group 52 (one strain), Chlamydia Research Group 1 (one strain), *Neochlamydia hartmanellae* (two strains), and *Endosymbiont acanthamoebae* UWE (two strains).

To explore the possibility that these *Chlamydia*-like strains were contaminants, we tested the water (Sigma) used in PCR mixtures, the water after passage of the Qiagen columns, and the elution buffer after passage of the columns. The addition of Sigma water to the PCR mixture of assay-C yielded no *Chlamydia* spp.-positive signal. Of the Qiagen column processed water samples, 6 out of 20 were *Chlamydia* spp.-positive, as well as 5 of 20 elution buffer samples.

Table 1. Primers and probes used in the PCR assays

Assay	Primers	Name	Probes	Target
A[a]	TGACAACTGTAGAAATACAGC	CpnA	*GACACACGTGCTACAATGGTT	Cp[b] 16S gene
	CGCCTCTCTCCTATAAAT	CpnB		
B[c]	TTACAAGCCTTGCCTGTAGG	oCP1		
	GCGATCCCAAATGTTTAAGGC	oCP2		
	TTATTAATTGATGGTACAATA	iCPC	*AGACTATGTTTTCGACCGTATCTTA	Cp OmpA gene
	ATCTACGGCAGTAGTATAGTT	iCPD		
C	GAGAATTTGATCTTRGTTCAG	CHLF	CGTCTAGGCGGATTGAGAG	Cspp[d] 16S gene
	*CCCTTTACGCCCAATAAATCC	CHLR-B	GAATGTAGTGTAATTAGGCATCT	Cp 16S gene
			AACGGAGCAATTGTTTCGA	Ct[e] 16S gene
D[f]	CGGCGTGGATGAGGCAT	16SIGF		Cspp 16S gene
	TCAGTCCCAGTGTTGGC	16SIGR		

*5'-Biotinylated. [a, c, f]References 17, 50 and 41, respectively. [b]Cp, *C. pneumoniae.* [d]Cspp, *Chlamydia* species. [e]Ct, *C. trachomatis.*

Table 2. PCR results in 66 vascular specimens by different PCR assays

	Single-step PCR		Nested PCR		PCR-based RLB[a]
No. of specimens	Assay-A1[b]	Assay-A2[b]	Assay-B1[b]	Assay-B2[b]	Assay-C[b]
18	-[c]	-	-	-	-
28	PCR+[d]	-	-	-	-
8	PCR+	-	-	-	Cspp+[e]
12	-	-	-	-	Cspp+
Total (n=66)[f]	36	0	0	0	20

[a]RLB, reverse line blot. [b]In assay-A1 and assay-B1, Taq Polymerase was used; in assay-A2 and assay-B2, AmpliTaq Gold polymerase was used; in assay-C, HotStarTaq DNA polymerase was used. [c]-, PCR was negative both in agarose gel electrophoresis and after hybridization. [d]PCR+, PCR was positive in agarose gel electrophoresis and negative after hybridization. [e]Cspp+, *Chlamydia* species-positive in RLB hybridization. [f]None of the specimens was *C. pneumoniae*-positive.

Discussion

The possible role of *C. pneumoniae* in the pathogenesis of vascular disease has been widely investigated. The DNA amplification of *C. pneumoniae* specific sequences in vascular tissue specimens however, has been shown to vary greatly between study groups. More recent reports have criticized the lack of standardization of *C. pneumoniae* PCR methodology [3, 4, 15].

In this study we investigated vascular tissue specimens of 66 patients by the PCR assays that have been most widely used [17, 50], and we explored the impact of different DNA polymerase enzymes on the results based on agarose gel electrophoresis and after hybridization. In addition, we investigated the presence of *Chlamydia* spp. DNA in the 66 samples, using a *Chlamydia* spp. PCR and RLB hybridization with both a *Chlamydia* spp.-specific and a *C. pneumoniae*-specific probe.

The rate of PCR-positive signals by gel electrophoresis in the single-step PCR depended on which DNA polymerase was used. When the conventional Taq DNA polymerase was used (assay-A1), 36 specimens (54.5%) were PCR-positive. If agarose gel electrophoresis had been the final method of detection in combination with the use of Taq DNA polymerase, 36 of 66 samples would have been labeled *C. pneumoniae*-positive in our study. These 36 samples however could not be confirmed by hybridization of the PCR products with a *C. pneumoniae*-specific probe. Also these samples were negative when Amplitaq Gold DNA polymerase was used. This may be due to the production of more specific products by Amplitaq Gold DNA polymerase than the conventional Taq DNA polymerase. DNA polymerase enzymes like Amplitaq Gold or Hotstart that need to be activated at elevated temperature are known to enhance the specificity of a PCR assay [27]. Reviewing the literature on the use of Amplitaq Gold DNA polymerase in *C. pneumoniae* PCR assays we found that in the majority of the studies, it was not mentioned which type of DNA polymerase enzyme was used. In a study that explicitly mentioned the use of Amplitaq Gold DNA polymerase, the detection rate of *C. pneumoniae* DNA was 14.8% [30].

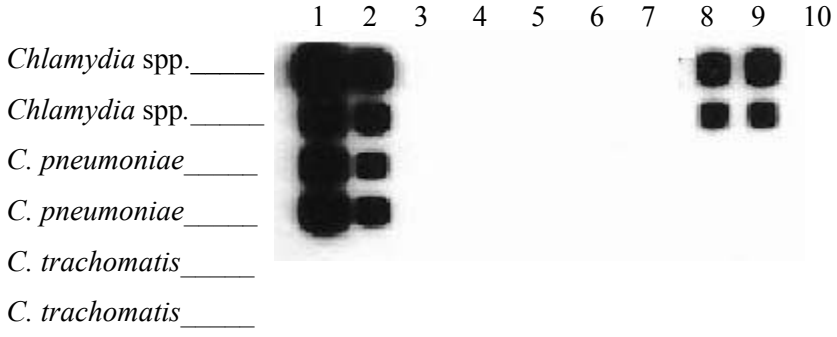

Figure 1. Part of the reversed line blot after hybridization with PCR products obtained from controls and clinical specimens. 200pmol and 100 pmol of *Chlamydia* genus-, *pneumoniae*-, and *trachomatis*-specific probes were spotted horizontally as indicated on the left. PCR products were hybridized vertically. Lane 1, 10 IFUs *C. pneumoniae* DNA*;* lane 2, 1.0 IFU *C. pneumoniae* DNA*;* lanes 3 and 7, negative controls; lanes 4-6 and 8-10, clinical specimens.

Hybridization is also an important measure to minimize false PCR-positive signals, and the advantages of confirmation of PCR-positive signals by hybridization with a specific probe have been described before [4]. Analyzing 33 studies with regard to hybridization in the detection of *C. pneumoniae* DNA, we found 11 studies in which only gel electrophoresis had been used to visualize PCR products [8, 16, 18, 30, 37, 38, 40, 42-45]. In the other 22 studies, PCR results were confirmed by hybridization [2, 5, 6, 12, 14, 19, 23-26, 28, 29, 31-35, 39, 46, 48, 49, 51]. The detection rate was significantly higher in studies with only gel electrophoresis than in studies with hybridization, 31.6% (204/645) and 24.5% (367/1492), respectively (P=0.0009). It is possible that the results of studies without hybridization are confounded by unspecific PCR-positive signals that are, incorrectly, interpreted *C. pneumoniae*-positive.

Using the *Chlamydia* spp. PCR and RLB hybridization we detected *Chlamydia* spp. DNA in 30% (20/66) of the specimens. Identification by sequence analysis of 6 of 20 PCR positive samples demonstrated the presence of *Chlamydia*-like organisms, including *Endosymbiont acanthamoebae* and *Neochlamydia hartmanellae*.

Chlamydia-like organisms may infect free-living amoebae that are common inhabitants of the aquatic environment. Amoebae may act as reservoir for these organisms, implying that *Chlamydia*-like organisms have potential for widespread dissemination [20]. They have been detected in nasal mucosa of healthy persons, in broncho-alveolar lavage of patients with respiratory tract infection, and in abdominal aneurysms [1, 13, 22, 41]. It has been demonstrated that elementary and reticulate bodies of *Chlamydia*-like organisms behave, in the life cycle, similarly to the chlamydial elementary and reticulate bodies [21]. In addition, the 16S rRNA sequence analysis of eight *Chlamydia*-like strains has been shown to be highly homologous with *C. pneumoniae,* varying from 85% to 87.6% [20]**.** The potential pathogenic role of *Chlamydia*-like organisms has not been established, and there is no evidence that *Chlamydia*-like organisms are associated with vascular diseases. Furthermore, it is possible that these strains were not originally present in the patients' specimens, and the positive PCR signals found might be a result of contamination with the environmental *Chlamydia*-like organisms [36, 41].

Indeed, we demonstrated the presence of *Chlamydia* spp. DNA by RLB in 11 samples after passage of the Qiagen columns, including elution buffer and distilled water. In the light of the homology between *Chlamydia*-like organisms and *C. pneumoniae*, one might hypothesize that sequence homology with *Chlamydia*-like organisms might be responsible for positive results in *C. pneumoniae* PCR assays. This may, in part, explain the inter- and intralaboratory discrepancy in the detection of *C. pneumoniae* in vascular tissue specimens.

In the nested PCR, all specimens were negative both by gel electrophoresis and by hybridization. Since the nested PCR was based on the sequence of the OmpA gene, a gene that usually shows more sequence variation compared to the 16S rRNA gene, no effect of the DNA polymerase was found in this assay. The disadvantage of the nested PCR is that no anti-contamination with dUTP/UNG can be used. Therefore it is most sensitive to contamination.

This study identified important factors that may have contributed to bias and once more shows the important influence of methodological factors on the detection of *C. pneumoniae* DNA in vascular tissues. In conclusion, we strongly recommend the use of DNA polymerases that have to be activated, the use of dUTP/UNG anti contamination, hybridization with specific probes, the inclusion of sufficient controls, the use of molecular grade water, and cautious interpretation of the results when column-based DNA extraction methods like Qiagen are used. This study furthermore stresses the biases that unstandardized methodology may introduce in the context of the possible link between *C. pneumoniae* and vascular diseases.

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Chapter 9

Effect of clarithromycin treatment on *Chlamydia pneumoniae* in vascular tissue of patients with coronary artery disease: a randomized, double-blind, placebo-controlled trial

Hans Berg, Boulos Maraha, Anneke van der Zee, Siska Gielis, Paul Roholl, Gert-Jan Scheffer, Marcel Peeters, Jan Kluytmans

Submitted

Abstract

Several, small clinical trials have indicated that antibiotic treatment of *Chlamydia pneumoniae* is associated with a better outcome in patients with coronary artery disease (CAD). It has not been demonstrated if antibiotic treatment eradicates *C. pneumoniae* from vascular tissue. The aim of this study was to assess the effect of clarithromycin on the presence of *C. pneumoniae* in vascular tissue of patients with CAD. Patients with CAD, waiting for coronary artery bypass graft surgery were enrolled into a randomized, double-blind, placebo-controlled trial. Patients were treated with clarithromycin 500 mg or placebo once daily, from the day of inclusion until surgery. During surgery several vascular specimens were obtained. The presence of *C. pneumoniae* in vascular specimens was examined by immunohistochemistry (IHC) and two polymerase chain reaction (PCR) assays. *Chlamydia* IgG titers were determined using an enzyme immunoassay, at inclusion and 8 weeks after surgery. A total of 76 patients were included and 180 vascular specimens were obtained (80 specimens from the clarithromycin group and 100 specimens from the placebo group). 35 patients received clarithromycin (mean duration 27 days, SD 12.2) and 41 placebo (mean duration 27 days, SD 13.9). IHC detected *C. pneumoniae* major outer membrane protein antigen in 73.8% of specimens in the clarithromycin group vs. 77.0% in the placebo group (p = ns). *Chlamydia* lipopolysaccharide antigen was found in one placebo specimen only. *C. pneumoniae* DNA was not detected in any specimen. Baseline *Chlamydia* IgG titers were equally distributed in both groups and were not significantly different after treatment. There is no indication of the presence of viable organisms in vascular tissue. Clarithromycin treatment of patients with CAD has no effect on the presence of *C. pneumoniae* in vascular tissue or on *Chlamydia* IgG titers.

Introduction

Many risk factors for atherosclerosis have been identified. However, atherogenesis is not fully understood and recently infectious pathogens, particularly *Chlamydia pneumoniae,* have been considered as potential risk factors for atherosclerosis [1]. It has been proposed that, during respiratory tract infection, *C. pneumoniae* reaches vascular tissue via infected leukocytes. In vascular tissue, *C. pneumoniae* can infect atheroma-associated cells and induce inflammatory cytokines production and smooth muscle cells proliferation [2]. *C. pneumoniae* may also cause endothelial dysfunction and promote the secretion of matrix-degrading metalloproteinases that destabilize the atherosclerotic plaque [2, 3]. Chlamydial lipopolysaccharide (LPS) and chlamydial heat shock protein 60 kd may contribute to atherogenesis by promoting foam cells formation, lipoprotein oxidation and proinflammatory activation [2].

Some seroepidemiologic studies have found an association between *C. pneumoniae* and coronary artery disease (CAD). Prospective serologic studies, however, failed to demonstrate any association [4]. Further indications that *C. pneumoniae* might play a role in atherogenesis

came from studies that identified *C. pneumoniae* in vascular tissue by polymerase chain reaction (PCR), immunohistochemical staining (IHC), electron microscopy, and culture [1]. However, the results of these studies are inconsistent and huge variations in detection rates are reported [1, 5].

The results of some small clinical trials that showed beneficial effects from antibiotic treatment encouraged many groups to further investigate the effect of antibiotic treatment on secondary prevention of cardiovascular events [6-8]. These studies are based on the hypothesis that antibiotic treatment of *C. pneumoniae* eradicates the organism from the vascular wall in CAD patients. This will end the infectious process, which will stabilize atheromas and decrease cardiovascular events. However, it has not been studied whether antibiotic treatment eradicates *C. pneumoniae* from vascular tissue of CAD patients. We initiated a placebo-controlled, double-blind, randomized clinical trial to investigate the effect of clarithromycin treatment on the presence of *C. pneumoniae* in vascular tissue, and on circulating *Chlamydia* IgG antibodies in patients with CAD.

Patients, materials and methods

Study population

Between July 1999 and July 2001, patients with documented CAD and scheduled for coronary artery bypass graft (CABG) surgery were invited to participate in the study. Inclusion was carried out during attendance at the pre-operative outpatient clinic at the department of thoracic surgery of the Amphia Hospital. Exclusion criteria included: (I) concomitant administration of terfenadine, rifabutin or cisapride; (II) antibiotic therapy with a macrolide, tetracycline, or quinolone within three months prior to inclusion or during the study period; (III) renal failure (serum creatinine rates above 150 μmol/l); (IV) elevated liver function tests (alanine-aminotransferase > 55 U/l, aspartate-aminotransferase > 45 U/l, total bilirubin > 27 μmol/l, or alkaline phosphatase > 180 U/l); (V) female patients capable of child-bearing but not taking adequate birth control precautions.

After giving informed consent, patients were randomized in a double-blind, placebo controlled trial. Patients received, from the day of inclusion until the day of surgery, a daily dose of clarithromycin 500 mg slow release (SR) or a placebo tablet (Clarithromycin SR and matching placebo tablets were obtained from Abbott laboratories, Abbott Laboratories Ltd, Queenborough, Kent, England ME11 5EL).

An independent pharmacist dispensed either clarithromycin or placebo tablets according to a computer-generated randomization table, which stratified in groups of 10. The researcher responsible for seeing the patients allocated the next available number on entry into the trial, and provided the patient the corresponding tablets. The code was revealed to the researcher once recruitment, data collection, and laboratory analysis were complete. The local Medical Ethics Committee approved the study.

Clinical specimens

During CABG surgery specimens were obtained from coronary atheromas, obstructed old coronary grafts, mammary artery, and saphenal vein, when possible. All specimens were divided into two portions, one for IHC and one for PCR. IHC samples were routinely fixed in 10% buffered formalin until further research. PCR samples were transported at 4 ºC in 200 µl of lysis buffer (1M Tris pH 7.0, 0.5 mM EDTA, 5M NaCl, 1% SDS, 20 mg/ml proteinase K), and processed within 24 hours.

From each patient 10 ml blood was obtained on the day of inclusion and 8 weeks after surgery. Blood was stored at 4 °C immediately after collection, and centrifuged within two hours. Serum was then stored at – 20 °C pending further testing.

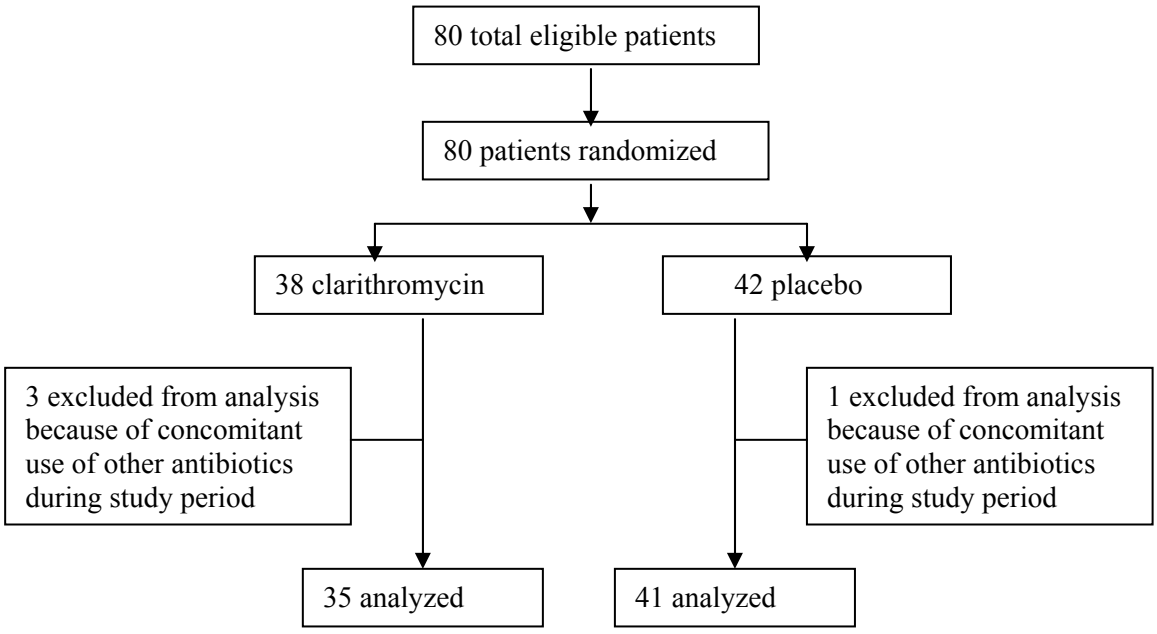

Figure 1. Trial profile.

Laboratory methods

Serology

Chlamydia IgG antibody titers were determined by a recombinant enzyme immunoassay (rELISA, Medac GmbH, Hamburg, Germany) according to the manufacturer's instructions. This rELISA uses a recombinant *Chlamydia*-specific LPS fragment as antigen. An IgG titer of $\geq$ 1:100 was considered positive.

Immunohistochemistry

Cross-sections of each paraffin wax embedded vascular specimen were stained with hematoxilin-eosin stain. In each cross-section, the lumen area, the circumference of the internal elastic lamina and the area encompassed by it, were evaluated. Antigens were

detected in 4 µm sections by IHC as described by Meijer et al. [9]. In the IHC, two monoclonal antibodies were used. The species specific monoclonal antibody RR-402 against *C. pneumoniae* major outer membrane protein (MOMP) (Washington Research Foundation, Seattle, Washington, USA) [10], and the *Chlamydia* genus specific anti-LPS monoclonal antibody 16.3B6 (produced by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands). HEp2 cells (CCL23; American Type Culture Collection) infected with *C. pneumoniae* strain TW-183 were used as positive control, and mock-infected HEp2 cells were used as negative control.

The specimens were evaluated microscopically by one experienced technician. Specimens were considered positive for *C. pneumoniae* antigen when a clear dot-like-cell associated staining was observed [5].

Polymerase chain reaction

Specimens processing

Within 24 hours after surgery, DNA was extracted from clinical specimens by the QIAamp DNA mini kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions. A control was included with every four clinical specimens in the extraction procedure.

Real-time PCR

A real-time PCR assay specific for *C. pneumoniae* and designed to the VD4 variable domain of the ompA gene was performed. Oligonucleotide primers included VD4 forward primer (5'-TCC GCA TTG CTC AGC C-3'), VD4 reversed primer (5'-AAA CAA TTT GCA TGA AGT CTG AGA A-3'), and a VD4 probe (5'-FAM-TAA ACT TAA CTG CAT GGA ACC CTT CTT TAC TAG G-TAMRA) [11].

To be able to monitor possible inhibition of PCR in the clinical specimens a universal internal control was used. This internal control sample consisted of a whole-virus preparation of phocid herpesvirus (PhHV-1) [12], which was added to the original clinical sample at a final concentration of approximately 5,000 to 10,000 DNA copies per ml. Primers PhHV-F1 (5'-GGG CGA ATC ACA GAT TGA ATC-3'), PhHV-R1 (5'-GCG GTT CCA AAC GTA CCA A-3') and probe (5'-VIC-TTT TTT ATG TGT CCG CCA CCA TCT GGA TC-TAMRA-3') were used to amplify PhHV1, which in uninhibited samples had a cycle threshold value of approximately 30. Amplification was carried out with both *C. pneumoniae* and PhHV1 specific primers and probes in a multiplex PCR.

Reactions were prepared with 96-well MicroAmp optical plate (Applied Biosystems) by addition of 5 µl of extracted DNA to 45 µl of PCR mixture containing 1x TaqMan universal PCR master mix (Applied Biosystems), 600nM VD4 forward primer, 300nM VD4 reversed primer, and 150 nM FAM fluorescent *C. pneumoniae* specific probe, 5 µl PhHV1 (whole-virus), 400 nM PhHV-F1 forward primer, 400 nM PhHV-R1 reversed primer, and 150 nM

VIC-labeled PhHV-1 specific probe. The 96-well plate was centrifuged for 1 min at 1,000 x*g* at room temperature in a swing-out rotor to remove small air bubbles in the reaction vessels.

Amplification and detection were performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) by using the manufacturer's standard protocols. The PCR cycling program consisted of 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15s at 95°C and 1 min at 60°C. Each run contained: (I) negative controls (one for every four extracted DNA samples); (II) positive control series containing a known amount of inclusion forming units (IFU) of *C. pneumoniae* (5, 2, and 1 IFU); (III) a negative mix control. A specimen was considered positive for *C. pneumoniae* if the fluorescence was above the threshold limit. Specimens were considered negative for *C. pneumoniae* if the internal control was positive with a cycle threshold value of $\leq$ 35.

Industry-developed research-use-only LCx *C. pneumoniae* PCR (RUO-PCR)

The presence of *C. pneumoniae* DNA in specimens was also examined by an Industry-developed LCx *C. pneumoniae* RUO-PCR (Diagnostics Division, Abbott Laboratories, Abbott Park, Chicago, Illinois, USA). The RUO-PCR assay was performed at Abbott Laboratories by Abbott personnel as described earlier [13]. Briefly, an activation mixture was prepared by mixing equal volumes of LCx Activation Reagent II and LCx *C. pneumoniae* Oligo Mix. 40 µl of the freshly prepared activation mixture and 30 µl of the purified DNA samples were subsequently added to the appropriate LCx amplification vial. The total reaction volume was 200 µl. Amplification was carried out with a 480 thermocycler (Perkin-Elmer, Norwalk, Conn.) under the following conditions: 97°C for 2 min; 40 cycles of 97°C for 30 s, 59°C for 30 s, and 72°C for 30 s; and finally, 1 cycle of 97°C for 5 min and 12°C for 5 min. PCR products were detected with the LCx Analyzer. Samples yielding a rate over 100 cps per second were considered *C. pneumoniae* positive. This cutoff was determined by testing titerated *C. pneumoniae* isolates and uninfected HEp-2 cell DNA multiple times [13].

Specimens were coded and all detection experiments were performed blind. The code was revealed when the study was completed.

Statistical analysis

All baseline characteristics were analyzed using a χ^2-test or a Student's *t* test when appropriate. A value of $P<0{\cdot}05$ was considered statistically significant. The SPSS 11·0 statistical software package was used for all calculations.

Role of the funding source

Abbott Pharmaceuticals supported this study with an unrestricted educational grant. Also, RUO-PCR was performed by Abbott Laboratories. The sponsor played no role in study design, data analysis, data interpretation, writing of the report, or in the decision to submit the report for publication.

Results

Figure 1 shows that a total of 80 patients with CAD, waiting for CABG surgery, were enrolled in the study. Four patients who during the study period concomitantly used other antibiotics were excluded from this treatment analysis due to possible additive effects. Thirty-five patients were randomly assigned to receive clarithromycin and 41 to receive placebo. Table 1 shows that the baseline patient characteristics are well balanced between the two treatment groups. The mean number of treatment-days, for both groups, was 27 as indicated by the number of tablets used.

Table 1. Baseline patient characteristics[a]

Characteristics	Clarithromycin, N = 35	Placebo, N = 41
Male	32 (91.4)	35 (85.3)
Age, mean	65 (8.6)	65 (9.3)
Weight, mean	84 (19.4)	81 (12.1)
Length, mean	170 (20.2)	174 (9.9)
NYHA-score, mean	3 (0.9)	3 (0.7)
Treatment-days, mean	27 (12.2)	27 (13.9)
Current smoker	5 (14.3%)	4 (9.8%)
Smoker in past	27 (77.1)	31 (75.6)
Medical history		
COPD	6 (17.1)	3 (7.3)
Diabetes Mellitus - Type I	2 (5.7)	1 (2.4)
- Type II	4 (11.4)	8 (19.5)
Hypercholesterolaemia	20 (57.1)	27 (65.9)
Malignancy	3 (8.6)	1 (2.4)
CVA or TIA	2 (5.7)	6 (14.6)
Pectoral angina	33 (94.3)	40 (97.6)
Myocardial infarction	20 (57.1)	23 (56.1)
Valvular insufficiency	3 (8.6)	1 (2.4)
Hypertension	18 (51.4)	25 (61.0)
Earlier vascular surgery	10 (28.6)	15 (36.6)
Family medical history		
Cardiovascular disease	29 (82.9)	31 (75.6)
Diabetes mellitus	7 (20.0)	6 (14.6)
Medication		
Statins	21 (60.0)	27 (65.9)
Anti-hypertensive drugs	35 (100)	41 (100)

Values are means (SD) or numbers (%); [a]Baseline patient characteristics are not significantly different between the treatment groups.

During CABG surgery, 180 vascular specimens were obtained, including coronary atheromas (n = 31), obstructed old CABG specimens (n=12), mammary artery specimens (n = 66), and saphenal vein specimens (n = 71). All atheromas and obstructed old CABG specimens showed histological signs of inflammation and advanced atherosclerosis (thickened intima, plaques with thrombus, lymfocytes, foamcells, surface defects). The other specimens showed normal to slightly thickened vascular walls.

The results of IHC are presented in table 2. *C. pneumoniae* MOMP antigen was found in the majority of specimens of both study groups. *Chlamydia* LPS antigen was found only once, in an atheroma from the placebo group. There was no significant difference in the presence of antigens between the two groups. The real-time PCR and the RUO-PCR failed to detect *C. pneumoniae* DNA in the 180 vascular specimens.

Chlamydia IgG antibody titers were measured on inclusion (baseline serology) and 8 weeks after completion of treatment. Baseline serology was positive in 81.6% and 73.8% of the patients in the clarithromycin group and the placebo group, respectively. The corresponding percentages 8 weeks after treatment were 78.9% and 66.7%, respectively. Clarithromycin had no significant effect on *Chlamydia* IgG antibody titers. In both treatment groups, *Chlamydia* IgG antibody titers after treatment were not significantly different compared with the baseline titers.

Table 2. Results of immunohistochemical staining of the vascular specimens from the study patients[a]

	Clarithromycin	Placebo	*P* value [b]
	Positive specimens/ specimens tested, (%)	Positive specimens/ specimens tested, (%)	
Coronary atheroma			
MOMP[c]	10/14 (71.4)	10/17 (58.8)	0.47
LPS[d]	0/14 (0)	1/17 (5.9)	0.36
Old coronary graft[e]			
MOMP	6/6 (100)	5/6 (83.3)	0.30
LPS	0/6 (0)	0/6 (0)	1.0
Mammary artery			
MOMP	22/28 (78.6)	30/38 (78.9)	0.97
LPS	0/28 (0)	0/38 (0)	1.0
Saphenal vein			
MOMP	21/32 (65.6)	32/39 (82.1)	0.11
LPS	0/32 (0)	0/39 (0)	1.0
Total			
MOMP	59/80 (73.7)	77/100 (77.0)	0.69
LPS	0/80 (0)	1/100 (1.0)	1.0

Values are numbers (%); [a]more than one specimen was obtained from some patients; [b]a p-value of < 0,05 is considered statistically significant; [c]MOMP, *C. pneumoniae* major outer membrane protein antigen; [d]LPS, *Chlamydia* lipopolysaccharide antigen; [e] Obstructed old bypass graft from earlier CABG procedure.

Discussion

Macrolide antibiotics, including clarithromycin, are active against *C. pneumoniae*. The hypothesis that *C. pneumoniae* is a risk factor for atherosclerosis has lead to clinical trials of macrolide treatment in patients with CAD [8]. Human placebo-controlled trials have been performed to investigate the clinical effects of antibiotics in patients with vascular disease. These studies follow the hypothesis that macrolides will kill *C. pneumoniae* in the vascular wall, which will subsequently end the infectious process. Thereby, the plaque will be stabilized which will result in fewer complications of atherosclerosis. However, whether antibiotic treatment has an effect on the presence of the microorganism in vascular tissue has not been studied well.

In the present study, vascular specimens were tested by an automated real-time PCR, which combines amplification, hybridization and quantitative product detection. Also, the specimens were tested by an industry-developed RUO-PCR. Both methods failed to detect *C. pneumoniae* DNA in any specimen. This indicates that there is no evidence of active *C. pneumoniae* infection in vascular tissue of CAD patients. This questions the use of antibiotics for this indication. Possible explanations for the negative results of the PCR assays could be the low DNA concentration in the test samples and the patchy distribution of *C. pneumoniae* DNA in vascular tissue [14].

Melissano et al. [15] evaluated the effect of roxithromycin on *C. pneumoniae* in carotid atheromas. The authors concluded that roxithromycin treatment was effective in eradicating *C. pneumoniae* from carotid atheromas since *C. pneumoniae* DNA was detected in 31% (5/16) of the atheromas in the roxithromycin group and in 75% (12/16) of the atheromas in the control group. However, they used a conventional semi-nested PCR assay to detect *C. pneumoniae* DNA. Conventional PCR assays are unstandardized and known to produce conflicting results, including false-positives [16, 17]. Also, this small trial was unblinded which limits the accuracy of their results. Efforts have been recently focussed on the establishment of quantitative real-time PCR and on RUO-PCR technology. The first reports on the evaluation of these tests suggest that they are sensitive, specific and reproducible [13, 18]. The conclusion from our DNA-detection experiments must be that there is no active *C. pneumoniae* infection in vascular tissue of patients with CAD.

The results from IHC were different. A high prevalence of *C. pneumoniae* MOMP antigen was found in both groups. *Chlamydia* LPS antigen was detected by IHC in only one specimen from the placebo group. These results are consistent with the findings of other investigators. The abundance of MOMP antigen and low detection rate of LPS antigen in vascular specimens has been reported before [1, 9, 22]. IHC is a valuable technique, which is limited by a subjective reading and interpretation. This remains difficult because of background staining and nonspecific staining with antigenic components in vascular specimens, such as inflammatory cells and tissue components [19-21]. It is also possible that components of

Chlamydia-like microorganisms described recently can cause cross-reactivity to the monoclonal antibodies used in IHC staining [9].

The conflicting results concerning the high detection rate of *C. pneumoniae* MOMP antigen and the low detection rate of *C. pneumoniae* DNA might be partly explained by the biology of chlamydiae [23]. Alternation of activity and latency characterizes infections with chlamydiae. In advanced chlamydiae infections, the pathogen is no longer present, whereas its antigens can persist for a long time. The persisting antigens may cause a cascade of events leading to fibrosis and scarring [23]. This proposed pathophysiology is in line with our findings.

In the present study, we could not demonstrate any effect of clarithromycin on the presence of *C. pneumoniae* MOMP antigen in vascular tissue. Similar finding has been demonstrated in animal models. Antibiotic treatment with azithromycin was not associated with elimination of chlamydial antigen from vascular specimens of rabbits infected with *C. pneumoniae* [24]. Also, in a mice model, azithromycin treatment did not affect the presence of *C. pneumoniae* in the aorta, lung, or spleen [25].

Clarithromycin treatment had no effect on *Chlamydia* IgG antibody titers, and we found no significant difference between baseline titers and the titers measured 8 weeks after treatment. Circulating *Chlamydia* antibody titers have been used as a marker of response to antibiotic treatment. Gupta et al. [6] reported a significant effect of azithromycin on *Chlamydia* IgG antibody titers. However, our study supports the results of other trials that have found that antibiotic therapy does not influence *Chlamydia* antibody titers in patients with vascular disease [26-30].

This is the first placebo-controlled, double-blind, randomized clinical trial that assessed the effect of antibiotic treatment on the presence of *C. pneumoniae* in vascular tissue. The major finding in this study is, that in vascular tissue, including atherosclerotic plaques of patients with advanced CAD, no viable *C. pneumoniae* are present. Only MOMP-antigen was found. Since viable *C. pneumoniae* are no longer present in vascular tissue, it is most unlikely that antibiotic treatment will have any effect in patients with advanced atherosclerosis. This explains the results of many clinical trials that failed to demonstrate any beneficial effect of antibiotic treatment in patients with vascular disease [26-32].

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Chapter 10

Discussion and summary

A link between infectious agents and vascular diseases has been proposed for decades. Since the well-defined risk factors for vascular diseases cannot explain all cases, attention has been recently focused on investigating hypothetical additional risk factors (chapter 1). The theory that inflammation contributes to the pathogenesis of vascular diseases has gained more support over the past few years. An association between elevated markers of systemic inflammation and vascular disease has been demonstrated [18]. However, several factors might be responsible for inflammation found in patients with vascular disease [12]. Microorganisms, especially *Chlamydia pneumoniae*, have been postulated as potential risk factors for vascular diseases. The first report of a possible association between *C. pneumoniae* and vascular disease came from a Finish group in 1988 [80]. Since then, a huge number of reports addressing the involvement of *C. pneumoniae* in the pathogenesis of vascular diseases has been published. These reports include seroepidemiologic studies, pathologic and molecular studies, animal model studies, and clinical antibiotic treatment trials. The data in these studies were obtained by microimmunofluorescence test (MIF), enzyme immunoassays (ELISA), polymerase chain reaction (PCR), immunohistochemistry (IHC), and sporadically by electron microscopy and culture. The studies presented in this thesis were performed to further investigate the possible link between *C. pneumoniae* and vascular diseases.

The association between *C. pneumoniae* and vascular diseases was initially based on serology. Three meta-analysis reports addressed this association. Eighteen early seroepidemiologic studies were analyzed by Danesh et al. [17]. Only one study failed to detect an association between vascular diseases and seropositivity to *C. pneumoniae*. The other 17 studies found odds ratios for association ranging from 1.2 to more than 8. However, only three of these studies were prospective. Three years later, Danesh et al. [19] analyzed 15 seroepidemiologic prospective studies. This analysis showed a weak association to no association at all, and the combined odds ratio of 15 studies for IgG was 1.5 (95% CI, 0.97-1.36). These findings were confirmed by a third meta-analysis that included 38 studies [10]. The cross-sectional studies demonstrated an association between *C. pneumoniae* seropositivity and vascular diseases, whereas in prospective studies no association was found [10].

In this thesis, the seroepidemiologic link between *C. pneumoniae* and vascular diseases was assessed by two case-control studies (chapters 3, 4, and 5). In a case-control study of patients with abdominal aortic aneurysm (AAA), we found inconsistent serologic results concerning the association between *C. pneumoniae* and AAA (chapter 3 and 4). We observed that serologic methods have an important implication on the serologic association between *C. pneumoniae* and AAA. Serology was performed by five different tests: two MIF tests (MRL-MIF and Savyon-MIF) and three ELISA's (Medac-rELISA, Savyon-ELISA and Bioclone-ELISA). Only in the MRL-MIF test, a significant association was found (odds ratio 6.8, 95% CI, 2.0-24). The other four tests showed no association. However, when the higher IgG titers were used as cutoff, also the MRL-MIF test failed to demonstrate any association between *C.*

pneumoniae and AAA. The link between low IgG titers of *C. pneumoniae* and AAA, demonstrated by the MRL-MIF test, might be a result of cross-reaction between the antigen used in the test and non-chlamydial components related to AAA, such as inflammatory cells and tissue components. Furthermore, the agreement between the five different tests was poor.

In order to investigate the hypothesis that *C. pneumoniae* might be responsible for inflammation associated with venous thrombosis, we evaluated *C. pneumoniae* seropositivity in a case-control study of patients with venous thrombosis (chapter 5). The results showed no association between *C. pneumoniae* and an increased risk for venous thrombosis. IgG antibody titers against *C. pneumoniae*, measured by a MIF test, were not correlated with the concentrations of the inflammatory mediators IL-6 and IL-8. This indicates that the inflammatory process shown in patients with venous thrombosis is not related to *C. pneumoniae* infection. Venous thrombosis is a multicausal disease and several genetic and acquired risk factors have been identified [79]. However, about one-third of episodes of venous thrombosis cannot be explained by the established risk factors. It has been suggested that inflammatory processes are involved in the pathogenesis of venous thrombosis [87]. However, the mechanism by which inflammation could be involved in this disease remains unknown. Our results do not support the hypothesis that *C. pneumoniae* infection is a risk factor for venous thrombosis. The link between *C. pneumoniae* and venous thrombosis was addressed in three other seroepidemiologic reports. These reports produced conflicting results. Two reports suggested that *C. pneumoniae* is associated with venous thrombosis [22, 52], whereas the third report did not confirm such an association [46].

Our serologic findings (chapters 3, 4, and 5) are consistent with the results of other studies. A multi-center study demonstrated interlaboratory inconsistency in *C. pneumoniae* serology measured by the MIF test [74]. Twenty-two identical sera were tested in 14 laboratories from 8 countries. The agreement with the reference laboratory ranged from 60% to 90%. A poor agreement between serologic tests for *C. pneumoniae* was also demonstrated in two recent studies evaluating the agreement between serologic tests in patients with vascular disease [32, 83]. Several factors may contribute to disagreement between *C. pneumoniae* serologic tests including the subjective interpretation, the test procedure, the type of the antigen used, its purity and its concentration [30]. Serologic tests for *C. pneumoniae* are not standardized. Commercial and in-house made serologic assays have not been adequately evaluated in patients with infection documented by culture [29]. In these assays different antigens are used, including purified elementary bodies (lipopolysaccharide (LPS)-extracted), purified major outer membrane protein (MOMP), and recombinant LPS-fragment. In addition, the definition of acute, chronic and past infection is based on different cutoffs and different criteria, depending on the serologic assays that are used. There is accumulating evidence that *C. pneumoniae* serologic tests are less specific than assumed. Unspecific reactivity in serologic tests, including cross-reactivity between *C. pneumoniae* and other *Chlamydia* species, has been demonstrated [30, 45, 63]. This probably is due to a lack of LPS removal from the

elementary bodies during antigen preparation. Unspecific serologic reaction may result from cross-reaction with the MOMP of different *Chlamydia* species or from cross-reaction with LPS [14, 73]. There have been also reports on a possible cross-reactivity to *Bordetella* pertusis and parvovirus [39, 59, 75]. The recently described new *Chlamydia*-like species belonging to three new families (*Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae*) in the order *Chlamydiales* may also cause serologic antigenic cross-reactivity with *C. pneumoniae*, since there is evidence that these strains display molecular cross-reactivity with *C. pneumoniae* [32, 71].

We observed a high seroprevalence of *C. pneumoniae* antibodies in healthy controls and in patients with AAA, venous thrombosis, and cardiovascular disease (chapters 3, 4, 5, 6, and 9). This is consistent with the findings of many seroepidemiologic studies that measured high seropositivity in patients with vascular diseases and in the general population [25, 32, 45]. We consistently defined positive serology as a marker of exposure to *C. pneumoniae*. However, seropositivity to *C. pneumoniae* has been interpreted in several ways and the definition of acute, chronic and persistent infection varied among reports [29]. The serologic criteria of *C. pneumoniae* infection are not standardized [12]. The Centers of Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) have recommended to use a fourfold rise in IgG titer or an IgM titer of $\geq$16 as criteria for acute infection [21]. An IgG titer $\geq$ 16 could indicate past exposure to *C. pneumoniae*, but no criteria for chronic or persistent infection could be formulated [21]. In addition, it has been suggested that a single high IgG titer has a poor predictive value [12]. In a study of healthy adults without symptoms or signs of infection, 17% fulfilled the criteria of *C. pneumoniae* acute infection (IgG $\geq$ 512 and/or IgM$\geq$ 16), while culture and PCR of respiratory specimens were negative [33].

In chapters 3 and 6, we found that *C. pneumoniae* serology does not correlate with the detection of *C. pneumoniae* by PCR and IHC. This finding confirms the poor agreement reported by the majority of the reports addressing this issue [29]. *C. pneumoniae* has been detected by PCR and IHC in seronegative patients, which might implicate false positive PCR and IHC or false negative serology, or may be due to the natural delay in immune response, or even to lack of immune response. On the other hand, *C. pneumoniae* culture-positive infection episodes without seroconversion have been reported, especially in children [29].

In addition to serology, studies have been designed to find evidence that *C. pneumoniae* is present in vascular lesions. The first study that investigated the presence of *C. pneumoniae* in atheromas came from South Africa [81]. The investigators found, by electron microscopy, *C. pneumoniae*-like microorganisms in the core of 7 atherosclerotic plaques. Five of these plaques were positive with *Chlamydia* genus-specific and *C. pneumoniae*-specific monoclonal antibodies in the immunohistochemical staining. Subsequently, a large number of studies on the detection of *C. pneumoniae* were performed using PCR and IHC. These studies reported

variable detection rate of *C. pneumoniae* in atheromas and normal vascular specimens [11, 12].

In the study presented in chapter 2, it was shown that the QIAamp DNA mini kit (Qiagen) is a useful and sensitive DNA extraction method for *C. pneumoniae* detection in vascular tissue. Compared with three other DNA extraction methods: NucliSens Kit (Organon), buffer-saturated phenol (Life Technologies), and Geneclean II Kit (Qbiogene), the purification of *C. pneumoniae* DNA with the QIAamp DNA mini kit was found to be the most sensitive. This purification was rapid and easy to perform. However, these findings should be interpreted with caution, since we were unable to create exactly reproducible dilution series of *C. pneumoniae* DNA. Moreover, it is not known whether the performance of the four procedures in the extraction of *C. pneumoniae* DNA from spiked materials is the same as their performance when used on patient materials. The QIAamp DNA mini kit and two other established DNA extraction methods: the High pure PCR template preparation kit (Boehringer) and the conventional phenol-chloroform protocol were compared in a multi-center study [4, 5]. Identical sets of dilution series of atheroma samples spiked with *C. pneumoniae* DNA, atheroma samples spiked with *C. pneumoniae*, and unspiked samples were tested in four laboratories. The results of that study suggested that the QIAamp DNA mini kit and the High pure PCR template preparation kit are equally sensitive, and superior to the conventional phenol-chloroform protocol. Mygind et al. [64] compared 5 DNA extraction methods using different types of samples. They used a real-time PCR for *C. pneumoniae* detection. The DNeasy Tissue kit (uses the same principle as the QIAamp DNA mini kit and both are manufactured by Qiagen) was the most sensitive of the five extraction methods when used for both pure DNA samples and aorta homogenates spiked with DNA, whereas the MagNA Pure method (Roche) was the most sensitive for purified elementary bodies and homogenates spiked with elementary bodies.

We reviewed the results of 22 studies on *C. pneumoniae* detection by PCR with regard to DNA extraction methods used. The QIAamp DNA mini kit was used in 7 studies [42, 43, 49, 57, 70, 76, 77], and variations of the conventional phenol-chloroform protocol were used in 15 studies [9, 20, 23, 36, 37, 53-56, 69, 72, 78, 82, 86, 90]. Studies in which the phenol-chloroform protocol was used to extract DNA obtained significantly more *C. pneumoniae* PCR-positive results than studies that have used the QIAamp DNA mini kit, 32.1% (399/1240) and 15.9% (47/295), respectively ($p< 0.05$). However, this does not necessarily imply that the phenol-chloroform is more sensitive than the QIAamp DNA mini kit, since there are many variables between the reviewed studies.

To further assess the link between *C. pneumoniae* and vascular diseases, we performed PCR and IHC staining to examine the presence of *C. pneumoniae* in clinical specimens. Peripheral blood cells (chapters 3 and 5) and vascular tissue (chapters 6, 8 and 9) obtained from patients with vascular diseases were tested.

The studies described in chapters 3 and 6 were initiated in 1998. In chapter 3, using a 16S based single-step PCR, *C. pneumoniae* DNA was detected in peripheral blood cells of 18 (20%) of 88 patients with AAA and 8 (9%) of 88 control subjects. The odds ratio for association was 2.9 (95% CI, 1.0-8.5), indicating an association between AAA and *C. pneumoniae*. The pathogenesis of AAA is not fully understood, but there is evidence that genetic factors are implicated in the development of AAA, which is supported by the familial clustering of AAA. Also, several proteolytic factors are considered as risk factors for AAA [41, 58]. Interaction between these risk factors probably promotes proteolytic activity in the arterial wall, which gives a rise to aneurysm dilatation. We hypothesized that *C. pneumoniae* might induce a chronic immunologic activation causing chronic endothelial cell damage and mediating a proteolytic process in the wall of the abdominal aorta [42]. Two previous studies on the association between AAA and *C. pneumoniae* had included control tissues. These reports provided conflicting results, Ong et al. [69] found no association, but Petersen et al. [76] showed a strong association.

We also report positive results in chapter 6. Using 16S based PCR, we detected *C. pneumoniae* DNA in 22% (10/45) of atheroma specimens and in 10% (5/50) of aortic specimens obtained from patients with cardiovascular disease. Since atheromas were significantly more frequently positive in PCR, we suggested that *C. pneumoniae* is associated with vascular disease and that *C. pneumoniae* might be involved in the development of atherosclerosis.

It has been suggested that in the context of the possible link between *C. pneumoniae* and vascular disease, also the association between vascular disease and other bacteria's such as *Mycoplasma pneumoniae* should be investigated [85]. This because the similarity in epidemiological behavior between *M. pneumoniae* and *C. pneumoniae* [85]. In chapter 7, we investigated the presence of *M. pneumoniae* in vascular specimens. Our results showed that *M. pneumoniae* is not associated with vascular disease. We tested atheromas and degenerative heart valve specimens by *M. pneumoniae* PCR. One (2.5%) of the 39 atheromas and two (3%) of the 64 degenerative heart valve specimens were PCR positive. These findings were confirmed in chapter 6, in that study *M. pneumoniae* was detected by PCR in one of 95 vascular specimens. The *M. pneumoniae* PCR assay used in these two studies (chapter 6 and 7) has been validated on respiratory samples and it had high sensitivity [34]. However, in the absence of the "gold standard" in the diagnosis of *M. pneumoniae*, the validation of in house made *M. pneumoniae* PCR remains difficult [51]. Quality control studies of *M. pneumoniae* PCRs have revealed both false-negative and false-positive results, indicating deficiency of these unstandardized methods [51].

Our later studies (chapter 5, 8 and 9) investigating the presence of *C. pneumoniae* in peripheral blood cells and vascular tissue were performed in 2000. In chapter 5, we performed PCR to detect *C. pneumoniae* DNA in peripheral blood cells of patients with venous thrombosis and control subjects. The detection rate of *C. pneumoniae* was very low in the

patients and the control subjects 0.5% (1/185) and 0.9% (2/220), respectively. These results confirmed the serologic findings that *C. pneumoniae* is not associated with venous thrombosis. The inconsistency in the detection rate of *C. pneumoniae* by PCR, between this study and our early study on AAA (chapter 3) is difficult to explain. In both studies the same PCR assay was performed on the same type of specimens (peripheral blood cells), but the detection rate varied substantially, 9% in the control subjects of the AAA study and 0.9% in the control subjects of the venous thrombosis study. We hypothesized that population differences between the two studies, such as the younger age of the subjects in the venous thrombosis study, and a poor reproducibility of the PCR assay might be responsible for the inconsistency in the detection rate.

Other investigators have also addressed the issue of inconsistency in the detection of *C. pneumoniae* by PCR. The rate of positive PCR varied among studies from 0% to 100% [11, 12]. Several possible explanations for this discrepancy have been suggested, such as population variations and insufficient blinding procedure [29]. However, there is accumulating evidence that variation in methods is the most plausible explanation [12]. *C. pneumoniae* PCR assays are not standardized and none of the available assays has been extensively evaluated compared to culture. Moreover, there is no "gold standard" assay that allows adequate interpretation of the obtained results [29].

Apfalter et al. [4, 5] addressed, in two multicenter studies, the interlaboratory inconsistency in the detection of *C. pneumoniae* DNA. In the first study [4], the agreement between 9 laboratories in the detection of *C. pneumoniae* was low, and the rate of positivity ranged from 0% to 100%. There was no consistency in pattern of positivity and the sensitivity of the assays used did not correlate with their detection rate. In addition, the negative control was reported positive in 19% of the assays. In the second study [5], the rate of positivity was higher among the negative controls than the vascular specimens. No single initially positive result could be confirmed after reamplification by a second PCR followed by hybridization with a *C. pneumoniae*-specific probe. They concluded that the reported variability in the detection of *C. pneumoniae* was caused by methodological factors rather than by differences in the prevalence of *C. pneumoniae* in the specimens [5]. It has also been shown that nested PCR assays yield more positive results than single-step assays [40]. We reviewed the results obtained by nested PCR or single-step PCR in 36 studies on the detection of *C. pneumoniae* in vascular specimens. In 23 studies, 1775 atheroma specimens were tested by a nested PCR assay and 497 (28%) specimens were positive [7-9, 16, 24, 49, 53-56, 65-67, 69, 70, 72, 76-78, 82, 85, 86, 90]. The 13 studies that performed a single-step PCR assay tested 460 atheroma specimens and detected *C. pneumoniae* DNA in 89 (19.3%) specimens [3, 13, 20, 23, 26, 36, 37, 42, 43, 47, 48, 57, 88]. The corresponding percentages for control tissue specimens were 10.7% (27/251) for the nested PCR and 3.5% (10/286) for the single-step PCR. Although, nested PCR assays produce more positive results, it has been shown that these assays have many limitations [5]. Nested PCR assays are prone to contamination and do

not allow the use of decontamination treatment with UNG [5]. Therefore, the reliability of the results of studies that performed a nested PCR assay has been questioned [5].

In chapter 8, we identified methodological factors that might contribute to intra- and interlaboratory inconsistency in the detection of *C. pneumoniae* DNA by PCR. We investigated the presence of *C. pneumoniae* in vascular specimens (61 atheromas and 5 AAA) by the PCR assay used in our previous studies (chapter 3, 5 and 6) and by a nested PCR. Also, we performed a 16S PCR-based reverse line blot assay to detect *C. pneumoniae* DNA as well as *Chlamydia* species DNA. All 66 vascular tissue specimens were *C. pneumoniae*-negative in the three PCR assays. In the single-step PCR, if agarose gel electrophoresis was the final method of PCR product detection in combination with the use of conventional Taq DNA polymerase, 54.5% (36/66) of the samples would have been accepted as *C. pneumoniae*-positive. All these samples, however, were negative after hybridization of the PCR products with a *C. pneumoniae*-specific probe. The PCR assay revealed negative results also when Amplitaq Gold DNA polymerase was used. The impact of the DNA polymerase might be explained by the fact that Amplitaq Gold DNA polymerase leads to the production of more specific products than the conventional Taq DNA polymerase. DNA polymerase enzymes like Amplitaq Gold or Hotstart that need to be activated at elevated temperature are known to enhance the specificity of PCR [44]. The use of Amplitaq Gold DNA polymerase in PCR assays has been seldom explicitly reported in studies on the detection of *C. pneumoniae*. Our results stressed the necessity of hybridization with a specific probe as an important measure to minimize false-positive PCR signals. Reviewing the results of 2137 specimens tested by PCR (chapter 8), we showed that the detection rate of *C. pneumoniae* in the literature is biased by the definition of a positive PCR. In studies that accepted gel electrophoresis signals as PCR-positive, the detection rate is significantly higher than in those that accepted hybridization signals as PCR-positive, 31.6% (204/645) and 24.5% (367/1492), respectively ($p< 0.05$). It is possible that the results of studies without hybridization are confounded by non-specific PCR-positive signals that are, incorrectly, interpreted as positive.

In the nested PCR (chapter 8), all specimens were negative both by gel electrophoresis and by hybridization. Since the nested PCR was based on the sequence of the OmpA gene, a gene that usually shows more sequence variation compared to the 16S rRNA gene, no effect of the DNA polymerase was found in this assay. Using the reverse line blot PCR (chapter 8), we detected *Chlamydia* species DNA in 30% (20/66) of the specimens. Sequence analysis of 6 PCR-positive samples demonstrated the presence of *Chlamydia*-like microorganisms, including *Endosymbiont acanthamoebae*, *Neochlamydia hartmanellae,* Chlamydia Research Group 52, and Chlamydia Research Group 1. We hypothesized that *Chlamydia*-like microorganisms might influence the results of *C. pneumoniae*-PCR assays, and they might contribute to the inconsistency in the detection of *C. pneumoniae*. We demonstrated the presence of *Chlamydia* species DNA by reverse line blot PCR in non-clinical samples, including elution buffer and distilled water, after passage of the Qiagen columns. This

suggests that it is possible that *Chlamydia*-like microorganisms were not originally present in the patients' specimens, but they might be introduced by contamination [60, 71]. *Chlamydia*-like microorganisms may infect free-living amoebae that are common inhabitants of the aquatic environment. Amoebae may present a reservoir for these microorganisms, implying that *Chlamydia*-like microorganisms have potential for widespread dissemination [27]. Moreover, the 16S rRNA sequence of *Chlamydia*-like strains has been shown to be highly homologous with *C. pneumoniae* 16S rRNA [27]. The negative results in this study (chapter 8) stress the inconsistency in the detection of *C. pneumoniae* at our laboratory, since in our initial study (chapter 6) *C. pneumoniae* DNA was detected by PCR in 22% of atheromas and in 10% of aortic specimens. It is noteworthy that our early studies on the detection of *C. pneumoniae* (chapter 3 and 6) provided positive results, whereas our subsequent studies (chapter 5 and 8) were negative. Although, the same PCR was used and all tests were performed at the same laboratory, there was a major variation in the detection rate between the early and the later studies. The results presented in chapter 8 suggest that DNA extraction, the type of DNA polymerase, anti-contamination with dUTP/UNG, and hybridization with specific probes influence the PCR results. Methodological factors are probably responsible for the inconsistency between the results of *C. pneumoniae* PCR assays. This indicates that the results generated by PCR assays, including our own results, are probably biased by a poor methodology.

It has been questioned whether the problem of *C. pneumoniae* PCR contamination can be controlled [5]. Therefore, more efforts have been recently focussed on the establishment of quantitative real-time PCR technology [6].

In chapter 9, we used two recently introduced techniques: a real-time PCR and an industry-developed research-use-only PCR assay. The real-time PCR requires less manipulation, resulting in lower risk of contamination; it is an automated and closed system; and it combines amplification, hybridization and quantitative product detection [6]. The industry-developed PCR research assay ensures consistent performance. Although the real-time PCR and the industry-developed PCR are not standardized, the first reports on the evaluation of these tests suggest that they are sensitive, specific and reproducible [6, 15].

The clinical trial described in chapter 9 was designed to examine whether clarithromycin treatment can eradicate *C. pneumoniae* from vascular tissue of patients with coronary artery disease (CAD). Patients with CAD, waiting for coronary artery bypass graft surgery were enrolled and randomly assigned to receive clarithromycin 500 mg or placebo once daily, from the day of inclusion till surgery. During surgery, vascular specimens were obtained and subsequently tested by a real-time PCR and an industry-developed PCR. These two PCR assays failed to detect *C. pneumoniae* DNA in any specimen. The negative results of the PCR assays indicate that there is no evidence for acute *C. pneumoniae* infection in vascular tissue of CAD patients. Other possible explanations for the negative results of the PCR assays could be the low DNA concentration in the test samples and the patchy distribution of *C.*

pneumoniae DNA in vascular tissue [16]. We were not able to demonstrate the effect of clarithromycin on the presence of *C. pneumoniae* DNA in vascular tissue, since all specimens were negative in the PCR assays. Melissano et al. [62] evaluated the effect of roxithromycin on *C. pneumoniae* in carotid atheromas. They found that roxithromycin treatment was effective in eradicating *C. pneumoniae* from carotid atheromas. An important difference between our study and the study of Melissano et al. is the high detection rate of *C. pneumoniae* DNA in their study. However, they used one unstandardized conventional semi-nested PCR assay to detect *C. pneumoniae* DNA. Taking the limitations of the design of the study of Melissano et al. (open and not placebo-controlled) into account, the reliability of their results might be questioned.

We used, as described in chapter 6 and 9, IHC staining to detect *C. pneumoniae* antigens in vascular specimens obtained from CAD patients. In chapter 6, we reported positive IHC staining for *C. pneumoniae*-MOMP in 60% (25/42) of the atheromas and in 9% (4/46) of the aortic specimens. In chapter 9, we used IHC to assess the effect of clarithromycin treatment on the presence of *C. pneumoniae* in vascular tissue of CAD patients. IHC detected *C. pneumoniae*-MOMP antigen in 73.8% of specimens in the clarithromycin group and 77.0% of specimens in the placebo group (p = ns). *Chlamydia*-LPS antigen was detected by IHC in one specimen from the placebo group. High detection rate of *C. pneumoniae* antigens by IHC has been reported by others [12]. However, protocols of performance and interpretation of IHC are not standardized [21]. IHC requires a subjective reading and its interpretation remains difficult because of background staining and nonspecific staining with antigenic components, such as inflammatory cells and tissue components, in vascular specimens [69, 82, 85]. It is also possible that components of *Chlamydia*-like microorganisms can cause cross-reactivity to the monoclonal antibodies used in IHC staining [60].

In chapter 6, we reported a statistically significant correlation between PCR and IHC in the detection of *C. pneumoniae* in atheromas. Unfortunately, we did not use established guidelines for the interpretation of agreement between tests [1]. According to these guidelines, the agreement between PCR and IHC found in our study (chapter 6) and most studies reviewed in chapter 6 is weak to fair. A good agreement is valid only for two of the reviewed studies [42, 82].

In the studies described in chapters 6 and 9, PCR and IHC produced conflicting results. Inconsistency between the results of PCR and IHC and the abundance of MOMP antigen and low detection rate of LPS antigen, in absence of *C. pneumoniae* DNA in vascular specimens has been reported before [60, 61]. This inconsistency might be partly explained by the biology of chlamydiae [31, 35]. Alternation of activity and latency characterizes infections with chlamydiae. In advanced chlamydiae infections, the pathogen is no longer present, whereas its antigens can persist for a long time. The persisting antigens may cause a cascade of events leading to fibrosis and scarring [31, 35].

The results of our clinical trial (chapter 9) indicated that clarithromycin treatment of CAD patients had no effect neither on the presence of *C. pneumoniae* in vascular tissue nor on circulating *Chlamydia* IgG titers. We concluded that antibiotic treatment of CAD patients is not indicated and it may not be beneficial, since *C. pneumoniae* infection in these patients is probably not acute and viable pathogens are no longer present in their vascular tissue.

A major finding in this study was, that in vascular tissues of patients with advanced CAD, no *C. pneumoniae* DNA could be detected, but only antigen debris of *C. pneumoniae*. In the light of these findings, it can be questioned whether an effect of antibiotics in patients with advanced atherosclerosis could be expected. This may explain the results of many clinical trials that failed to demonstrate any beneficial effect of antibiotic treatment in patients with vascular disease [2, 28, 38, 50, 68, 84, 89].

In conclusion, the results of this thesis do not support the hypothesis of a causal relationship between *C. pneumoniae* and vascular diseases. Antibiotic treatment has no effect on the presence of *C. pneumoniae* in vascular tissue of patients with advanced CAD. The available diagnostic methods of *C. pneumoniae* infection lack sufficient reliability and standardization. The results of *C. pneumoniae* PCR assays are inconsistent. The agreement between the results of *C. pneumoniae* serologic assays is poor. These limitations have important implications on the assessment of the possible role of *C. pneumoniae* in the pathogenesis of vascular diseases. The perceived association between *C. pneumoniae* and vascular diseases is influenced and probably biased by methodological factors. Further efforts should focus on optimizing and standardizing diagnostic methods of *C. pneumoniae* infection.

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Samenvatting

Chlamydia pneumoniae behoort, volgens de nieuwe classificatie van de familie *Chlamydiaceae*, tot het genus *Chlamydophila*. Binnen het genus *Chlamydophila* worden 6 species onderscheiden: *pneumoniae, psittaci, pecorum, abortus, felis* en *caviae*. Het genus *Chlamydia* heeft 3 species: *trachomatis, suis* en *muridarum*. Volgens de "oude" classificatie had de familie *Chlamydiaceae* één genus: het genus *Chlamydia*. In dit proefschrift, hanteren wij de oude benaming *Chlamydia pneumoniae* omdat deze beter bekend is onder de microbiologen en de klinici. Chlamydiae zijn op te vatten als Gram-negatieve bacteriën, die als gevolg van hiaten in hun metabolisme niet in staat zijn tot zelfstandig extracellulair bestaan en daardoor gedwongen zijn tot intracellulair parasitisme. Chlamydiae zijn gevoelig voor bepaalde antibiotica, o.a. voor macroliden en tetracyclines.. *C. pneumoniae* veroorzaakt pneumonie, bronchitis, pharingitis en sinusitis. De meeste kinderen raken op jonge leeftijd geïnfecteerd met *C. pneumoniae*. Op volwassen leeftijd (>50 jaar) zijn bij 60-80% van de mensen antistoffen tegen *C. pneumoniae* aantoonbaar.

Verschillende risicofactoren zijn betrokken bij het ontstaan van atherosclerose, het belangrijkste proces in coronair vaatlijden. Hypercholesterolemie, hypertensie, diabetes mellitus en roken zijn geïdentificeerd als factoren die een rol spelen in de pathogenese van atherosclerose. Echter, deze risicofactoren kunnen het voorkomen van coronair vaatlijden alleen bij 50-70% van patiënten verklaren.

Aneurysma van de aorta abdominalis (AAA) is een gelokaliseerde chronische dilatatie in de aorta, die ontstaat als gevolg van afbraak van extracellulaire matrixeiwitten en vervolgens van elastine- en collageenvezels in de aortawand. Genetische en familiare factoren zijn betrokken bij het ontstaan van AAA, echter de pathogenese van AAA is niet helemaal opgehelderd.

Veneuze trombose wordt gekenmerkt door trombosevorming als gevolg van endotheelcelbeschadiging, stase en verhoogde stollingsactiviteit. Verworven en genetische factoren spelen een rol bij het ontstaan van veneuze trombose. Echter, bij eenderde van de patiënten blijft de pathogenese van veneuze trombose onverklaarbaar.

Potentiële risicofactoren voor vaatziekten, zoals een mogelijke rol van chronische infecties, kregen in de afgelopen decennia veel aandacht. Finse onderzoekers vonden eind tachtiger jaren een associatie tussen een hoge antistoffentiter tegen *C. pneumoniae* en coronair vaatlijden. Sindsdien is er wereldwijd veel onderzoek gedaan naar een mogelijke rol van *C. pneumoniae* bij vaatziekten, zoals coronair vaatlijden, AAA en veneuze trombose. In de studies die beschreven zijn in dit proefschrift wordt de associatie tussen *C. pneumoniae* en vaatziekten onderzocht.

In hoofdstuk 2 worden vier verschillende procedures voor de extractie van *C. pneumoniae* DNA uit vaatwandmonsters vergeleken. De onderzochte procedures zijn: NucliSens, QIAamp DNA MiniKit, buffer-saturated phenol, en Geneclean II. In dit onderzoek hebben wij 30 bioptiemonsters van de aorta gebruikt. De resultaten lieten zien dat de QIAamp DNA MiniKit een gemakkelijk uit te voeren procedure is, met de hoogste gevoeligheid van detectie.

Hoofdstukken 3 en 4 beschrijven een patiënt-controle onderzoek naar de relatie tussen *C. pneumoniae* en AAA. In dit onderzoek werden perifere bloedcellen en serummonsters van 88 patiënten met AAA en 88 gezonde personen onderzocht. Met polymerase kettingreactie (PCR) werd *C. pneumoniae* DNA vaker gedetecteerd in perifere bloedcellen van patiënten met AAA dan bij gezonde personen. De serologische resultaten waren inconsistent. Omdat de serologische "gouden standaard" test voor *C. pneumoniae* ontbreekt, hebben wij de antistoffen tegen *C. pneumoniae* bij patiënten en controles met 5 verschillende serologische testen bepaald. Slechts in een van de 5 testen (MRL-MIF), werden antistoffen tegen *C. pneumoniae* vaker aangetroffen bij de patiënten dan bij de gezonde personen. Deze bevinding kon echter bij een hoog afkappunt van de MRL-MIF (IgG$\geq$512) niet bevestigd worden. In de andere 4 serologische testen werd geen significant verschil tussen AAA patiënten en controles gevonden. Bovendien was de overeenstemming tussen de resultaten van de 5 testen in het algemeen zwak. Op basis van de PCR resultaten werd geconcludeerd dat *C. pneumoniae* geassocieerd kan zijn met AAA. Deze associatie werd niet ondersteund door de serologische resultaten, en was afhankelijk van welke serologische test werd gebruikt. Geconcludeerd werd dat de beschikbare, niet gestandaardiseerde serologische testen, de resultaten met betrekking tot de associatie tussen *C. pneumoniae* and AAA beïnvloeden. Deze bevinding verzwakt de hypothese die veronderstelt dat *C. pneumoniae* een rol kan spelen in de pathogenese van AAA.

In hoofdstuk 5 wordt ingegaan op de vraag of *C. pneumoniae* een rol kan spelen bij het ontstaan van veneuze trombose. Ook wordt de betrokkenheid van *C. pneumoniae* bij de inflammatie (IL-6 en IL-8) die geassocieerd is met veneuze trombose bestudeerd. Er werd een patiënt-controle onderzoek uitgevoerd, waarbij de patiënten recidiverende veneuze trombose hadden. Voor dit onderzoek waren perifere bloedcellen en serummonsters van patiënten (n = 185) en controles (n = 220) beschikbaar. Zowel een serologisch als een moleculair verband tussen veneuze trombose en *C. pneumoniae* kon niet worden aangetoond. De aanwezigheid van *C. pneumoniae* antistoffen was niet geassocieerd met een verhoogd risico van veneuze trombose. *C. pneumoniae* DNA werd gedetecteerd bij één patiënt en twee gezonde controles. De concentraties van IL-6 en IL-8 bij patiënten en controles waren niet geassocieerd met *C. pneumoniae* antistoffentiter. Wij concludeerden dat *C. pneumoniae* waarschijnlijk geen rol speelt bij het ontstaan van veneuze trombose, en dat *C. pneumoniae* niet verantwoordelijk is voor de ontstekingsreactie die geassocieerd is met veneuze trombose.

In hoofdstuk 6, 8 en 9 werd de relatie tussen *C. pneumoniae* en atherosclerose bestudeerd. In hoofdstuk 6 beschrijven wij een onderzoek waarbij atherosclerotische coronaire vaatwandmonsters en macroscopisch gezonde aortawandmonsters werden afgenomen tijdens vaatchirurgie bij patiënten met ernstig coronair vaatlijden. De aanwezigheid van *C. pneumoniae* DNA of *C. pneumoniae* membraaneiwit (MOMP) werd in deze monsters met 2 methoden onderzocht, PCR en immunohistochemie (IHC), respectievelijk. Ook werden de antistoffen tegen *C. pneumoniae* in serummonsters van deze patiënten bepaald. *C.*

pneumoniae DNA en MOMP werden in de atherosclerotische vaatwandmonsters vaker gedetecteerd dan in de aortawandmonsters. Deze resultaten suggereren dat er een verband bestaat tussen *C. pneumoniae* en atherosclerose. De overeenstemming tussen de serologische resultaten en respectievelijk de PCR en IHC resultaten was zwak. Een zwakke tot matige overeenstemming was ook gevonden tussen de resultaten van PCR en IHC.

In hoofdstuk 7 gaan we in op de hypothese die veronderstelt dat *Mycoplasma pneumoniae* een plausibele kandidaat kan zijn om een rol in de pathogenese van atherosclerose te spelen. Om dit te onderzoeken hebben wij 39 atherosclerotische coronaire vaatwandmonsters en 64 degeneratieve hartklepmonsters, van patiënten die operatief zijn behandeld, onderzocht met *M. pneumoniae* PCR. *M. pneumoniae* DNA werd gedetecteerd in één atherosclerotisch vaatwandmonster en in 2 degeneratieve hartklepmonsters. Daarom kan geconcludeerd worden dat *M. pneumoniae* is niet geassocieerd met atherosclerose en zeer waarschijnlijk geen rol speelt in de pathogenese van deze ziekte.

In hoofdstuk 8 wordt de invloed van methodologische factoren in PCR op de detectie van *C. pneumoniae* DNA in vaatwandmonsters beschreven. Vaatwandmonsters (61 atheromas en 5 AAA) werden afgenomen bij 66 patiënten met vaatlijden en werden getest met 3 verschillende PCR methoden, een ‘reverse line blot’ PCR, een ‘single-step’ PCR, en een ‘nested’ PCR. De laatste 2 PCR testen werden met twee verschillende DNA polymerase enzymen uitgevoerd. Dit onderzoek heeft aangetoond dat de methodologie een belangrijke invloed op de detectie van *C. pneumoniae* DNA heeft. Het type DNA polymerase, anticontaminatie met dUTP/UNG, hybridisatie met een specifieke probe, en DNA extractie met columns werden geïdentificeerd als methodologische factoren die de resultaten van de PCR kunnen beïnvloeden. Er werd geconcludeerd dat de associatie tussen *C. pneumoniae* en vaatziekten wordt vertekend door de niet-gestandaardiseerde methodologie.

In hoofdstuk 9 wordt het effect van clarithromycine op de aanwezigheid van *C. pneumoniae* in vaatwandmonsters en op *Chlamydia* IgG antistoffentiter in serum bij patiënten met coronair vaatlijden bestudeerd. Daarvoor hebben wij een placebo-gecontroleerd, dubbel blind, gerandomiseerd interventie onderzoek verricht. Patiënten met coronair vaatlijden (n = 76) die op de wachtlijst voor ‘coronary artery bypass graft’ operatie stonden, kregen tot de dag van de ingreep, eenmaal daags clarithromycine SR 500 mg of placebo. Tijdens de ingreep werden vaatwandmonsters afgenomen. Bloedmonsters werden bij inclusie en 8 weken na de ingreep afgenomen. De vaatwandmonsters werden met 3 methoden getest: IHC, ‘real-time’ PCR en ‘industry-developed research-use-only’ PCR. Er was geen significant verschil tussen de twee onderzoeksgroepen in de detectie van *C. pneumoniae* membraaneiwitten. Ondanks het gebruik van twee PCR methoden werd *C. pneumoniae* DNA in geen vaatwandmonster gedetecteerd. Clarithromycine had geen effect op de *Chlamydia* IgG antistoffentiter. Er werd geconcludeerd dat er geen aanwijzingen zijn voor levende *C. pneumoniae* in de vaatwand bij patiënten met coronair vaatlijden, en dat de behandeling met clarithromycine bij deze patiënten geen effect op *C. pneumoniae* heeft.

Het ontbreken van gestandaardiseerde methoden voor de detectie van *C. pneumoniae*-DNA, -antigenen, en -antistoffen is een complicerende factor in de evaluatie van de rol van *C. pneumoniae* in de pathogenese van vaatziekten. Methodologische factoren beïnvloeden de resultaten van seroepidemiologische en detectieonderzoeken, en als gevolg daarvan kan een mogelijke associatie tussen *C. pneumoniae* en vaatziekten vertekend worden.

De bevindingen van de studies beschreven in dit proefschrift ondersteunen niet de theorie van een causale relatie tussen *C. pneumoniae* en vaatziekten. De associatie tussen deze bacterie en vaatziekten is inconsistent. Antimicrobiële behandeling heeft geen invloed op de aanwezigheid van *C. pneumoniae* in de vaatwand bij patiënten met coronair vaatlijden. Verdere studies moeten gericht zijn op de optimalisering en standaardisering van *C. pneumoniae* diagnostische methoden.

Publications

Dankwoord

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Curriculum vitae

Boulos Maraha, geboren op 2 maart 1962 te Kamechli, Syrië.
Getrouwd met Kinda en samen hebben zij een zoon, Paul.

1997 artsexamen, Universiteit Maastricht
1998 aanvang opleiding specialisme Medische Microbiologie,
St. Elisabeth ziekenhuis te Tilburg. Opleider dr. M.F. Peeters.
2002 inschrijving als arts-microbioloog in de Medische Specialisten Register.
2002- arts-microbioloog, Regionaal Laboratorium Medische Microbiologie,
Dordrecht/Gorinchem, met als verzorgingsgebied: Albert Schweitzer ziekenhuis te Dordrecht, Beatrixziekenhuis te Gorinchem, huisartsen, verpleeghuisartsen, verloskundigen en GGD in de regio Dordrecht/Gorinchem.

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